



British Technology Group

Commons committee shows no mercy

A FIERCE attack on the British Technology Group (BTG) was launched this week by the House of Commons Public Accounts Committee (PAC). The chief criticism centres on the investment by the National Enterprise Board (with the National Research Development Corporation one of the two components of BTG) in the company called NEXOS, set up in January 1979 to develop and sell an integrated office automation system. PAC complains that it is "far from satisfied" with the explanation of how the company lost £31 million before it went into receivership in October 1981, and asks that the Department of Trade and Industry should carry out an investigation which "we shall wish to examine".

PAC says it has been hampered in its investigation of the collapse of NEXOS Office Systems Ltd by the fact that the Auditor and Comptroller-General does not have access to the accounts of the National Enterprise Board (NEB). It has, however, been able to establish to its own satisfaction from evidence given by officials that NEXOS, intended to market an office automation system based on word processors, facsimile machines and voice systems, failed because of over-optimistic projections of its sales, "unsatisfactory" relations with component suppliers and "management deficiencies".

The PAC report explains that NEXOS when founded was dependent on a commercial agreement with Logica VTS to be the sole source of supply of a word processor which had not been developed when the company first began trading and which was eventually put into production after the date first promised. PAC says that NEXOS "was obliged to purchase, effectively under a cost-plus contract", a word processor over whose development and quality it had no control, and under a contract that did not specify what should happen if "something went wrong".

PAC says that the trading loss reported for the period of NEXOS's existence of £17 million seems "extraordinarily high" by the yardstick of the company's best sales of £8.9 million in 1981, the year in which it collapsed. The committee also asks "whether any consideration was sought in respect of the £5.2 million loss" quoted for research and development and asks why £0.6 million of public money was written off under the heading of "bad debts".

Apart from its particular interest in this investment, PAC emphasizes that the NEXOS collapse is another reason why BTG should be made more directly accountable to the British Parliament. The report is likely to play an important part in

the British Government's legislation for a formal merger of NEB and NRDC, which has been planned for the past two years. Evidence given to PAC confirms that the unified organization is likely to function more as a sponsor of innovation along the lines of NRDC than as a source of public money to be used as venture capital, the chief role of NEB in recent years.

PAC is also, however, critical of BTG's handling of INMOS, the company set up in 1976 to manufacture silicon chips in the United Kingdom. Acknowledging that the venture was a "high-risk" undertaking, PAC says it is disappointed that sales

forecasts have consistently been unfulfilled but seems even more dismayed that the company's plan to employ 4,000 people by the mid-1980s has not yet been achieved, and that employment at Colorado Springs in the United States is still greater than that in Britain.

The future of BTG will no doubt be clouded by these opinions. The formal merger of the two component organizations apparently waits on parliamentary time (and the government's stomach for the argument it will provoke), but by all accounts BTG has now submitted its corporate plan to the Department of Trade and Industry, on which basis it is likely that the government will outline for BTG the manner in which it will be allowed to continue. One matter still unresolved is the promise, made at the Prime Minister's seminar on technology last September, that NRDC will relinquish its first refusal of innovations arising in the public sector. □

British universities

Tenure principle to be trimmed

THE British Government seems to have hit on the one device for the abolition of tenure in British universities that is likely to satisfy British academics. The Secretary of State for Education and Science, Sir Keith Joseph, announced last week plans to set up a "Statutory Commission" whose function will be to amend university charters to remove from academic contracts of employment explicit references to the expectation that academic appointments will normally continue until "normal retirement age".

The first reactions of university vice-chancellors to the proposal are generally speaking those of people fearful of being about to be hanged who have had their sentences commuted to life imprisonment. For the past four years, the British Government has made no secret of its distaste for the principle of tenure, and two years have passed since the Committee of Vice-Chancellors and Principals put forward proposals for amending the standard conditions of tenure. In particular, the committee suggested that probationary periods for new academics should be lengthened, and raised the possibility of periodic reviews of individuals' performance.

In a letter to the committee last week, Sir Keith Joseph complained that "with one or two limited exceptions, there has been no response" and said that the problem is probably one that the universities cannot "resolve by themselves". His letter also, however, makes plain that the government's objective is that only new contracts should provide for "reasons of redundancy or financial exigency".

The vice-chancellors, who saw the government's letter for the first time at their meeting last Friday, are relieved because the threat of blanket legislation explicitly governing the conditions of

academic employment has thus been withdrawn. Even so, the government will have to obtain an Act of Parliament delegating power to legislate to commissioners, whose task will be to rewrite university charters in line with the terms of reference eventually embodied in the act.

Meanwhile, the new development will provide academic lawyers with a rare opportunity for archival work. The proposed statutory commission will be the first since that which, in the early 1920s, arranged to bring the universities of Oxford and Cambridge within the umbrella of public support by giving the central administration of the two universities the right to receive funds from the then new University Grants Commission. Two earlier commissions (in the 1850s and 1870s) had similarly been concerned with the affairs of Oxford and Cambridge.

The enforced reduction of academic staffs in British universities in the past three years appears, so far, to have been accomplished without the principle of tenure being challenged directly. Part of the explanation appears to have been the relatively generous conditions on which it has been possible for academics over the age of 50 to retire early, but it seems clear that the academics' representative organization, the Association of University Teachers, has been anxious to avoid confrontation for fear of having to hazard the principle of tenure.

The universities seem also to have won a victory in their war of attrition with the government by securing an undertaking that a proposed investigation of the efficiency of academic administration, to be conducted at six universities not yet chosen, will not investigate the conduct of academic affairs. This inquiry is likely to be completed in two years. □

Japanese computers

Fifth-generation machines here

Tokyo

JAPAN'S fifth-generation computer project has taken a big step towards its preliminary goals with the successful construction of a prototype relational database machine — the first of its kind.

The fifth-generation computer project aims at the development, by the 1990s, of a completely new form of computer that can communicate in everyday language, "reason intelligently" and bring enormous amounts of stored knowledge to bear on any problem that the user might set it. The machines are intended to be cheap and reliable enough to be used in homes and offices. Some talk as if they will revolutionize society by making freely available a huge fund of expertise that will vastly "amplify human intelligence".

When the project was announced by the Ministry of International Trade and Industry (MITI) in 1981, its sheer boldness and originality astonished people in the West. Japan, considered weak in fundamentally new ideas, seemed overnight to have produced a plan that would give it an unassailable lead in advanced computer technology. One expert even uncharitably described the plan as a "technological *Mein Kampf*".

Since then, Western governments have responded more rationally with new projects of their own. There are, for example, advanced information technology projects in the United Kingdom, France and West Germany, together with the European Communities' Esprit programme, while the schemes of the United States' Defense Advanced

Research Projects Agency were directly inspired by Japan.

The fifth-generation computer will be composed of three main functions: an intelligent interface allowing it to communicate flexibly with humans by speaking, writing or drawing; problem-solving and inference functions allowing problems to be solved by deductive and inductive inference as well as by making sensible guesses where knowledge is incomplete; and a knowledge base function able systematically to store and retrieve not only vast quantities of data but also judgments and test results.

It is within this third function that 45 researchers at the Institute for New Generation Computer Technology (ICOT), set up by MITI to run the project in cooperation with Japan's big eight electronic manufacturers, have now announced a major step forward. Success with the construction of the relational database machine follows by five months the completion of the hardware of one of the other two essential parts of a prototype fifth-generation machine — a sequential inference machine.

The novelty in ICOT's machine is that relational software has been replaced by specially developed hardware. Not all the control software is complete so the machine is not actually doing anything yet. But when in operation, it will be the first true relational database "machine", working two orders of magnitude faster than anything else available. Named Delta, the device consists of two parts: a relational processor complex containing pipelined

relational algebra engines (built by Toshiba) and a hierarchical memory subsystem (built by Hitachi), plus an interface (built by Oki). The aim is to be able to connect it with the prototype sequential inference machine by next spring. ICOT hopes then to have two-thirds of a prototype fifth-generation machine, providing the programming environment in which to tackle the really big problems ahead.

Connecting the two together requires that both operate on the chosen "kernel" language of extended PROLOG, the advanced logic programming language invented in France and developed in the United Kingdom. There are several tricky software problems to be solved.

That both ICOT's sequential inference machine, which broke new ground as hardware by allowing extended PROLOG to be used as its machine language (see below), and the relational database centre on relations between things is no accident. ICOT's visionary director, Kazuhiro Fuchi, sees the predicate logic they use, established by Godel in the 1930s, as the key to building knowledge information processing systems. Indeed, he has said that the present generation of computers, based on the Turing theory published in 1936, may one day be seen as a "gigantic historical detour".

The fifth-generation computer project is scheduled to last ten years. The first three years, up to next spring, seem likely to achieve the goal of a prototype machine to be used as a base for further research. Eventually, however, machines will have to work at incomparably greater speeds than at present. The goal is 100 to 1,000 million logical inferences per second (Lips), compared with just 30,000 Lips for the present machine.

This speed can be achieved only by switching to some sort of parallel architecture for the sequential inference machine, probably based on a data flow model (in which the University of Manchester is an acknowledged leader). The project also has to solve the stupendous problems of developing syntactical analysis systems allowing communication with humans in natural language, making a complete revision of PROLOG for parallel use, developing knowledge-base machines utilizing parallel operations and pattern information processing systems.

What has been achieved so far consists of the rapid and determined development of ideas well researched elsewhere. The four-year intermediate stage of the project beginning in 1985, however, requires radical innovations — on time. Those years will decide whether a Japanese fifth-generation computer will emerge in the 1990s or whether it will have to be postponed until the world of information technology is much further advanced.

This year's budget for the project is ¥5,100 million (£16 million). Later budgets, it is said, will depend upon "progress in research". **Alun Anderson**

Logic and language for the new generation

RELATIONAL databases are not in themselves new — their mathematical basis was laid out in 1972. What makes them distinct from more conventional data bases, and particularly suits them to "intelligent" applications, is their simplicity of organization. Entries consist purely of relations (essentially two-dimensional tables).

This provides access to any datum in the base either directly or through association and allows data sets to be rearranged in new combinations. If, for example, a set of statistics about university students and their examination results is stored in a relational data base, it is easy to ask the base to provide, say, the names of all students who scored exactly 85 per cent in their examinations. Traditionally, this would be accomplished only by searching through the whole data base. The facility to retrieve data flexibly (by relational algebra) has obvious advantages for intelligent operations, for it will never be clear in advance just how knowledge will be used.

PROLOG, the chosen kernel language,

was originally devised for solving problems that involve symbolic representations of objects and relationships between them. To the average programmer, accustomed to writing sequential instructions, PROLOG is something of a surprise.

Thus programmes are written in terms of facts, for example:

Tim likes doughnuts
written as
likes (Tim, doughnuts)

rules, for example:

Tim likes any object
provided it is sweet
and questions, for example:
Does Tim like doughnuts?
written as:

? likes (Tim, doughnuts)

which will cause a search to be made to see if a precisely matching declaration can be found in the data. Building from these very simple roots, extremely complex logical arguments can be advanced and complicated inferences be made from one statement to another. **Alun Anderson**

Nuclear test fallout: 1

US Government arraigned

Washington

A FEDERAL judge in Utah has ruled that fallout from nuclear bomb tests was the "proximate cause" of certain types of cancers developed by nearby residents, and that the government had failed to take proper precautions. Ten plaintiffs, who had developed thyroid cancer, breast cancer or leukaemia, were awarded a total of \$2.7 million in damages. All but one of the ten have since died from their illnesses. The judge ruled that another 14 plaintiffs, who developed other forms of cancer, had not proved a link to the fallout.

Although the decision is certain to be appealed by the government, it has given encouragement to thousands of others who are claiming injury from exposure to low-level radiation generated by the government's nuclear programme. These include 375 other residents of southwestern Utah whose cases have not yet come to trial; uranium miners, who say they were not advised of the risks of radiation exposure; workers at the Nevada Test Site; sheep ranchers in Utah who claim their flocks were killed by fallout; and "atomic veterans" who served at Hiroshima and Nagasaki in Japan and at nuclear test sites.

The judge's decision rested heavily on a finding of negligence on the government's part in failing to carry out a proper monitoring programme that could have measured radiation levels in nearby areas and in failing to warn residents to recommend simple precautions. The plaintiffs lived approximately 100 miles

from the Nevada Test Site during the 1950s and early 1960s when above-ground nuclear testing was carried out. The plaintiffs were able to sue under a special law that allows findings of negligence against the government in certain cases.

In establishing the nuclear tests as the most likely cause of the cancers, the judge accepted the conclusions of several controversial studies that found higher than normal cancer rates in the region. A 1979 study by Dr Joseph Lyon of the University of Utah, cited in the ruling, found nearly twice the incidence of leukaemia in children exposed to the fallout as compared with children who were born long enough before testing began to escape exposure. That study, however, has been strongly criticized, most recently by Dr Charles Land of the National Cancer Institute; Land argues that the rate of the exposed group is actually no higher than the normal rate of Utah, and suggests that the lower rate of the earlier group may be the result of under-reporting of the disease at a time when that remote part of Utah had relatively poor health services.

The three cancers that the judge said were linked to the nuclear tests are the most sensitive to radiation induction. The plaintiffs received radiation doses of, typically, a few rads. According to several independent experts, these doses — particularly in the case of the leukaemias — are enough to attribute a significant though not overwhelming probability of causation to the nuclear tests.

Stephen BudianskyNuclear test fallout: 2

Claim and counter-claim

Canberra

THE federal Australian Labor Government is under growing pressure from its own backbenches and the Labor premier of South Australia, Mr John Bannon, to furnish more information about alleged secret nuclear tests at Maralinga in 1963, the allegedly unmarked burial of radioactive equipment and the storage of British tactical nuclear weapons on Australian soil during the 1958–61 partial test ban. It is also alleged that human guinea pig experiments were conducted during the seven official tests at Maralinga in 1956–57. Former civilian and ex-military workers on the test sites have risked prosecution under the Official Secrets Act to come forward with accounts — some anonymous, some in part mutually corroborative — that suggest unacknowledged nuclear test operations. These include stories of badly burned military personnel "berserk with pain", civilian workers told to "climb ladders" or to remain unwittingly in areas five miles away, during the tests carried out in the 1950s, and of fireball and mushroom-cloud explosions observed in 1961.

These claims follow the death-bed allegations by a former Royal Air Force technician, Mr John Burke, that he had found four aborigines dead in a bomb crater after one of three previously undisclosed tests in 1963. His claim to have informed the Commonwealth police of the deaths has provoked charges of an official cover-up, since no record has yet surfaced. Mr Burke died on 1 May of stomach cancer which he believed could be directly attributed to the tests.

Claims by the UK Ministry of Defence (MoD) that the three 1963 implosion trigger experiments were one-ton chemical blasts involving polonium may explain the aborigine deaths, but not, perhaps, the extent of the damage reported in the 1961 blasts, in which steel constructions are said to have been oxidized and lead bricks fused at a distance of one mile. The MoD "confirmation" that the 1963 tests were extremely minor sits uncomfortably with the recently-leaked unexpurgated version of the Pearce report on residual contamination at Maralinga, in which 20 kilograms of plutonium is reported to have been spread over four Maralinga sites during 1956–63, resulting in plutonium plumes of above-tolerance radioactivity extending over thousands of hectares.

The anecdotal eyewitness reports are at present unsubstantiated, but South Australian aborigine leaders say they are planning international court action and Mr Bannon has written to Mrs Margaret Thatcher, the British Prime Minister, and Mr Neil Kinnock, the British Opposition leader, for full disclosure.

Jeffrey Sellar

Government veto for Warsaw rector

THE Polish Ministry of Science, Higher Education and Technology has refused to confirm in office the newly elected rector of Warsaw University, Dr Klemens Szaniawski. This decision runs counter to pledges given through the government press spokesman, Mr Jerzy Urban, that university officers elected in accordance with the 1982 Higher Education Act would be confirmed in office "with a possible exception". The exception Urban referred to, however, was Dr Janusz Onyszkiewicz, the mathematical logician recently elected to the Warsaw University senate who had been the chief press spokesman of Solidarity. Dr Szaniawski, however, was not even a member of Solidarity.

The swift response from the ministry on Dr Szaniawski is surprising. According to the procedure laid down in the 1982 act, the minister had two weeks to reach a decision. In the matter of Dr Onyszkiewicz's election to the senate (a post far less significant for the running of the university), a stalemate has been allowed to develop. The ministry notified its objection to Onyszkiewicz's election (on the grounds of electoral

"improprieties") at the end of March. The outgoing rector, Dr Kazimierz Dobrowolski, said that he was working on his reply and then said he could not act further because he was unclear whether he should convene the old or new senate.

By vetoing the election of the new rector within three days, the ministry has clearly indicated its commitment to the slogan enunciated by Politburo member Tadeusz Porebski, at the plenary meeting of the new Association of Polish Students. The party and state leadership, he said, is "determined to counteract the capture of management posts [in education] by people who do not guarantee the socialist character of the school". Earlier official warnings had said only that the authorities could not tolerate university elections being used to secure a power base for "opposition" elements.

Dr Szaniawski, however, is an essentially moderate figure who, during the Solidarity era, acted as a mediator between the interests of the party and the demands of Solidarity in the drafting of the new higher education and censorship bills. Vera Rich

Biochemistry in Japan

Applying fundamentals

Tokyo

INCREASED support for fundamental research in the life sciences is planned in a report to Prime Minister Yasuhiro Nakasone by Japan's top science policy-making body, the Council for Science and Technology.

Concern about the long lead of the United States in basic research in molecular biology and the effect this will have on the commercial exploitation of genetic engineering lies behind the report, and swift action is expected on its main recommendations — increasing the numbers and mobility of young researchers and strengthening the technical back-up for biotechnology. But university researchers are likely to be disappointed, for although the report is entirely concerned with "fundamental" research, the government sees this as "essential research fundamental to planned applications". Expansion of truly fundamental research in the universities is unlikely.

To prepare the report, the council, which includes the heads of the science and finance-related ministries and agencies as well as industrial leaders, took special advice from both a life sciences group drawn from government, universities and industry and from a special committee comprising 22 of Japan's leading molecular biologists. Nakasone is expected to accept the report within a few weeks, when the ministries will be expected to work out how it might best be implemented. The financial implications should be included in ministry draft budgets by the end of the year.

Professor Hiuga Saito, head of the Tokyo University's Applied Microbiology Institute, who was the chairman of the council's special committee, stresses that the most important point made by the report is the need to give more young people a training in biotechnology, perhaps by means of special fellowships which would allow young scientists to carry out original research — a possibility already under consideration by the Ministry of Education, Culture and Science (MESC).

The report particularly emphasizes that all three of Japan's research groupings — the universities, government research laboratories and industry — must be encouraged to work together more closely. The idea of a special research institute, supported jointly by government and business, where young people could be trained, is floated. But the university side shows little enthusiasm for that project, and the chances are that it will proceed no further.

A more promising proposal is the removal of restrictions that hamper original research. Increased flexibility is, for example, to be encouraged in the research system so that research proposals not fitting into the conventional "project" funding can find support. Increased

freedom for young people to carry out individual research, and increased flexibility in the supply of equipment, perhaps by arranging for the rental of expensive equipment, are other possibilities.

University workers may gain from the recognition in the report that the pace at which biotechnology is advancing means that scientists can stay on top only if they can circulate around top laboratories — increased exchange of researchers is called for, both within Japan and between Japan and foreign countries, including more visits from foreign scientists.

The report also calls for the government to step up efforts to secure the basic resources of biotechnology. Calls are made for the creation of a larger collection of microorganisms, including those with special characteristics and from unique environments, as well as increasing stocks of cell lines, particularly those useful for the creation of hybridomas.

More rapid and thorough dissemination of data, particularly DNA and protein sequences but also data on the special characteristics of organisms and their life cycles, is seen to be important. A new computerized database to complement those existing elsewhere may result. Finally the report argues for the provision of more sophisticated tools for molecular biology — automatic DNA and protein synthesizers

and analysers, and microinjection and laser techniques.

If all these measures are put into effect, the result should be a major fillip for research in industry but not for the universities. What the universities lack at present is not money for molecular biology but permanent research staff. An increase in staff (or the new college or department of life sciences that Professor Saito would like to see) is, however, almost impossible under the present administrative system because the government has a long-term commitment to reduce the number of civil servants — and university researchers are civil servants, employed by MESC.

Industry can expect to benefit quickly from an increased flow of researchers and resources. Indeed, confidence that industry can do its own biotechnology research and apply that research with superb efficiency is expressed in a report from a private advisory body, the Japan Economic Research Centre, published after the government council's report. The report predicts that Japan will be the world leader in biotechnology by the end of the century: joint ventures and technical links with foreign companies will bridge the gap over the next three to five years and then Japan's proven ability to commercialize technology should enable it to begin to move into the lead. The only note of caution behind such predictions is the proviso that the universities will have to provide a good supply of trained personnel in search of jobs.

Alun Anderson

Molecular sequences

UK community seeks access

BRITISH molecular biologists, frustrated by inadequate computing facilities, are attempting to persuade research councils and cancer charities to finance a new national computer link-up and software support scheme for protein and DNA sequencing. Their problems will be aired at a special meeting next month of the Inter-Research Council Coordinating Committee on Biotechnology with interested parties.

The stimulus for the meeting was a letter from prominent researchers addressed to the three research councils that support molecular biology and to the Imperial Cancer Research Fund and the Cancer Research Campaign. The signatories were Dr Michael Ashburner of the University of Cambridge, Professor William Shaw of the University of Leicester and Dr David Glover of Imperial College, London. Their concern arose from a feeling in the research community that scientists have to waste too much time in writing or modifying programmes that are system-dependent. It was also felt there was scope for an improvement in access to databases.

Research council officials are at pains to emphasize that no financial commitment is implied by next month's meeting.

Enthusiasts in the research community, however, are already talking fondly of a network of "maybe 6 or 8" VAX machines at national centres, providing regular updates for databases and allowing the transfer of the latest software. These might in turn be connected to microcomputers for local analysis once the letter crunching had been done by the large machines. One estimate puts a price of "one or two million" pounds on the scheme.

The three research councils involved, the Medical Research Council, the Agricultural and Food Research Council (AFRC) and the Science and Engineering Research Council, all have their own budgetary problems. But, it is pointed out, all of these bodies give a high priority to the new biology, and the cancer charities are thought likely to look favourably on the idea. One of the difficulties is that the subject falls between the concerns of several research councils; however, the existence of the inter-research committee, whose chairman is Dr John Ingle of AFRC, has allowed a quick response. And, it is argued, the advantages of a computer link-up have already been demonstrated by the "Starlink" network used by astronomers.

Tim Beardsley

Peace protests

Soviet missiles
unwelcome

THE Czech human rights organization, Charter-77, two weeks ago formally repudiated views it alleged had been ascribed to it by the British-based "European Nuclear Disarmament" movement (END). Peace, said the Chartists, is not in itself a sufficient goal — it may mean little more than passivity in the face of tyranny. International peace, they said, can be guaranteed only if governments treat their own citizens with respect. Charter-77 is not a peace movement; there is a "long road" from "the absence of war" to "real peace".

The Chartists' statement seems an over-reaction to the current Warsaw Pact propaganda which puts the entire blame for the present nuclear confrontation on the United States. The Czechoslovak people as a whole seem reluctant to accept the official line. Party activists have reported difficulty in convincing factory meetings of the essential difference between "aggressive" United States missiles and their Soviet counterparts now being installed in Czechoslovakia to "defend peace".

Many have signed petitions against Soviet missiles, and students at the Charles University in Prague, aware that signing the petition would almost certainly result in expulsion, devised their own form of protest; a huge placard condemning the Soviet missiles was set up near the entrance to the university, with an invitation to all who agreed with the message to draw a little Sun on it. Within a couple of hours, the whole board was reportedly covered in Suns.

There is now evidence that concern about the Soviet missiles is widespread throughout the bloc. In East Germany, which is also to accommodate the Soviet missiles, there is a well-established "independent" peace movement. This accepts the concept of the bilateral withdrawal of missiles (and later of conventional arms), but is not acceptable to the authorities.

And in both Hungary and Bulgaria, the governments have denied widespread rumours that Soviet missiles may be arriving in the near future. The Bulgarians are particularly incensed, as they are committed to the campaign for a Balkan nuclear-free zone. In Romania (except when Soviet visitors are present), even official spokesmen take the line that they cannot apportion blame, albeit with hints that the West must make the first move. No "unofficial" opinions have so far been reported.

Poland has so far contributed little to the missile debate — save for the usual spate of "spontaneous" condemnations of the West. At least one group, however, has refused to sign such a document (see *Nature* 22 March, p.305). **Vera Rich**

Toxic chemicals

UK guidelines a compromise

BRITAIN'S Health and Safety Executive seems to have pleased nobody with its first attempt at British guidelines on occupational exposure to toxic substances, published last week under the unassuming title "Guidance Note EH 40". Some of the concentration limits specified are admitted by industry not to be achievable in the foreseeable future, while trade unions are complaining that the new document weakens their members' legal protection.

Until last week, Britain relied largely on the toxic chemical guidelines produced annually by the American Conference of Government Industrial Hygienists. These were used by factory inspectors to assess compliance with the statutory requirement on employers to ensure their employees' health, safety and welfare "so far as is reasonably practicable". But differences of practice between Britain and the United States, and the conflicting requirements of European Community directives, made home-grown guidelines desirable, and consultations were started in 1980.

The main change is that short-term exposure will in future be assessed over a 10-minute period, rather than (impossibly) instantaneously. This has pleased those who worry about the theory of occupational hygiene, even though, for substances with very rapid effects, the 10-minute principle will have to be ignored. But the numbers in the new list are copied wholesale from the US list it was intended to replace.

These "recommended limits" are "considered to represent good practice" and will be used by factory inspectors "as part of their criteria for assessing compliance". The Health and Safety Executive explains that there are not enough toxicity data on most substances to warrant "control limits", which have been drawn up for fewer than a dozen materials. Control limits for these materials have been set after prolonged haggling between employer and union representatives on the executive's Advisory Committee on Toxic Substances. Embarrassed by the shortness of the list of control limits, executive officials stress that EH 40 will be updated regularly.

Control limits, as distinct from recommended limits, are considered to be "reasonably practicable" and "should not normally be exceeded", according to the executive. But Mr Edward King, an ex-president of the Institute of Occupational Hygienists, says that the existing control limited for lead could not be achieved at any secondary lead smelting factory in Britain in the foreseeable future without unrealistic expense. The Chemical Industries Association reluctantly concedes that its members may have difficulty in reaching the supposedly enforceable control limit within 2 years. The limit for acrylonitrile may present similar

difficulties.

The Association of Scientific, Technical and Managerial Staffs (ASTMS) condemns EH 40 for being out of date, based as it is on the US list for 1980, which has since been updated. Others think this is no bad thing. ASTMS also complains that it has not been given documentation of the new limits — although there does not seem to be any new scientific work underlying EH 40 and the union is represented on the committee that fixed the limits.

So what is the professional occupational hygienist's considered view of EH 40? Mr King has no hesitation in describing it as window-dressing that will do nothing to improve anyone's health. He believes that most damaging exposure to toxic industrial chemicals now occurs during cleaning and maintenance work, when normal codes of practice are often informally suspended in any case. Real improvements will have to wait until factory inspectors are more numerous or can impose more effective sanctions than at present. **Tim Beardsley**

Modest ecological
disaster discussed

Washington

WHAT is fondly known as the "doom and gloom" lobby — the environment and world-future think-tanks that have been warning us for years about overpopulation, deforestation, acid rain, soil erosion and sundry depletions of non-renewable resources — met in Washington last week to say that things are not so bad after all. A conference organized by the World Resources Institute (a new "Center for Policy Research" in the Brookings Institution mould that is also known as the Council-on-Environmental-Quality-in-exile, thanks to the Reagan Administration's efforts to do away with that agency) entitled "The Global Possible" concluded that while the "doom and gloom" scenarios "could be valid", they were "confident that these trends can be reversed". How? By holding the world population to 8,000 million, arranging for a smooth transition to non-fossil fuels, controlling tropical deforestation, avoiding a disruption of global climate, increasing agricultural production, decreasing soil erosion, and, last but not least, strengthening public support for sound resource management.

Among the participants were Russell Train of the World Wildlife Fund, Lester Brown of the Worldwatch Institute, Martin Holdgate of the UK Departments of Environment and Transport and Mihajlo Mesarovic of Case Western Reserve University, a founding member of the Club of Rome.

Stephen Budiansky

Computerized dictionary

English language quantified

THE *Oxford English Dictionary*, generally known as the OED, plans to leap into the twentieth century by investing £7 million in the computerized production of a new edition, a merger of the existing 12-volume dictionary with the four-volume supplement still not yet complete. Announcing this plan earlier in the week, Oxford University Press said that the merging of the OED and supplement into a single printed work (to be published in 1988) is merely the first phase of an ambitious programme leading to the availability of the dictionary in an electronic form and eventually to the use of computers for novel lexicographical purposes.

Oxford seems to have been able successfully to turn the respect and affection in which the OED is held into tangible support for the computerization project. IBM (UK) Ltd is lending for the first phase of the project computer hardware (a 4341 processor and up to a score of terminals), software and the services of two specialists on secondment. This assistance, valued at £1 million over the duration of the first phase, compares with the £6.5 million in cash and kind committed in a year by IBM to the support of computer development projects in the United Kingdom.

The Department of Trade and Industry has also provided a grant of £288,750 from its fund called Support for Innovation. The donkey work of "data capture" (or keyboarding) will be carried out on a commercial basis by International

of which might be sufficient to contain the 1,000 million characters in the new edition. Oxford will pay a royalty for its use of the software, which the University of Waterloo will be free otherwise to exploit.

For users of the dictionary, the chief disappointment will be that the new edition, to be called the *New Oxford English Dictionary*, will be innocent of the entries accumulated since the appearance of the first supplementary volume nearly twenty years ago. Keyboarding will begin in September this year, but between now

and then the dictionary staff will be chiefly concerned to develop the means by which the entries in the OED and the four supplements can be accurately logged and merged, given the variety of typefaces and alphabets used and the need for subjective judgement in telling how supplemental entries supplement the originals.

As things are, the project is cautious about the extent to which the resource being computerized may be used as an automatic source of novel dictionaries, but there seems a good chance that scholars will eventually be able to use the dictionary as a means, for example, of monitoring the rate at which nouns are being converted into adjectives or even verbs. □

Nuclear power

UK peers give Europe advice

THE European Commission should play a stronger role in environmental and energy policy-making, according to a new British report from the House of Lords Select Committee on the European Communities. The committee concludes there is a clear need for an authoritative international assessment of safety and other environmental aspects of nuclear power aimed at reassuring public opinion. It suggests that the commission might join forces with a body such as the International Energy Agency, in order to avoid the appearance of making propaganda.

In the meantime, it suggests that European reliance on the pressurized water reactor could have the "grave disadvantage" that a serious fault or an accident in one of these reactors might cause public opinion to swing against the nuclear energy programme in general. Because of the need to support diverse energy sources, their lordships are concerned to protect and encourage the role of nuclear power, so they suggest that the EEC should give serious consideration to advanced gas-cooled reactors (AGRs) as a possible future design. One of Britain's AGRs, at Hinkley Point in south-west England, is described as "a model of efficiency".

The House of Lords Committee reserves some scornful words for the present policies of the European Commission's Directorate-General XI, which concerns itself with environment, consumer protection and nuclear safety: "Public concern — well-intentioned but not always well-informed — leads to regulatory action before the relevant facts have been established". The Commission directive on sulphur emissions is cited as an example.

The general European objective of reducing oil imports is endorsed, however. The committee sees a danger that lower oil prices at present, which it sees as only temporary, might provide member states with an excuse to delay the move away from dependence on oil. It is also worried by the prospect of greatly increased

imports of coal into the EEC, which are thought likely to be necessary in the 1990s. It therefore says that "profitable home production of coal should be encouraged", a delicately-worded statement that might be taken up by either side in the current British miners' strike.

Tim Beardsley

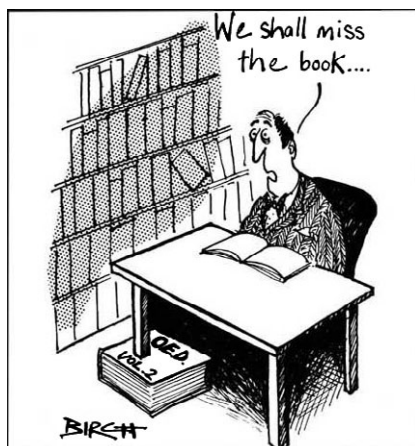
AFRC cuts meet disapproval

BRITISH Members of Parliament have taken up the cause of scientists employed by the Agricultural and Food Research Council who are threatened by redundancy. A report from the House of Commons Agriculture Committee published last week chides the government for imposing on the council cash limits that have forced a programme of contraction.

The committee's predecessor in the last parliament recommended a shift towards more food research, a move since implemented by the council. But the new committee now protests that it was never intended that this should be at the expense of mainstream agricultural research.

The council's submission to the Advisory Board for the Research Councils on scientific opportunities for 1985-86 includes a similar request for more provision for food science without this being at the expense of other work. Plant biotechnology is another area where the council wants to see increased provision.

The government's response to last year's Agriculture Committee report was to propose a new priorities board to advise on the strategy for scientific research related to agriculture, though in the words of Mr Michael Jopling, the Agriculture Minister, "it is expected the advice will usually be followed". But this does not satisfy the Public Accounts Committee, which complains that agricultural research is still "dangerously poised between many pinnacles of bureaucracy". Tim Beardsley



Computaprint of Fort Washington, Pennsylvania.

In preparation for the later phases of the project, Oxford has also made an arrangement with the University of Waterloo, Toronto, Canada, which has undertaken to develop software for the computerized handling of lexicographical data. One obvious possibility is that the dictionary, supplemented by recently accumulated material, will be made available on magnetic tape or by means of a data bank, but there is also a possibility of making the whole work available on optical disks, one

Nuclear war models

SIR — Our study of frost rings as indicators of climatically effective volcanic eruptions (*Nature* 307, 121; 1984) was discussed by John Maddox in the same issue (p.107) in the context of recent calculations modelling the long-term consequences of nuclear war. We appreciate his synopsis of our study but we do not necessarily agree with his conclusions on the applicability of the frost ring-eruption relationship to the "nuclear winter" question. Mr Maddox emphasized the formation of a frost ring as evidence of a short-lived climatic response to a major eruption and contrasted this seemingly moderate reaction to an atmospheric aerosol veil with the severe picture presented in the nuclear winter scenario, attributing the disparity between the two responses to the nature of the assumptions made about the quantities of dust carried into the stratosphere.

Frost rings are most likely to form at the end of a summer that is already notably or even abnormally cool. Cold delays the completion of cambial activity, rendering the tree more vulnerable to damage by late summer polar-air outbreaks, which occur while the tree is still growing. This suggests not only a short-term regional climatic response, in the form of an unusual incursion of cold air over a period of a few days in years following a major eruption, but also reflects a longer-term response of general cooling — an extreme example of which seems to have occurred in the North-Atlantic sector in the "year without a summer", following the eruption of Tambora in 1815. If a single point source of atmospheric aerosols injected into the stratosphere can have such significant apparent short-term and long-term effects on climate, as well as a characteristic biological response, we believe the implications for the response to global nuclear war are grave indeed.

Nevertheless, the volcano-climate link is appropriate as an analogue for only that portion of the nuclear-war scenario that occurs as a result of large amounts of dust and aerosols reaching the stratosphere. One should indeed expect a large disparity between the respective responses of the climate system to the aftermath of a major volcanic eruption and the aftermath of a global nuclear war, as the former is a reaction to conditions primarily in the lower stratosphere while the latter is likely to be a far more complex response to conditions through several layers of the entire atmosphere, the most important of which is probably cooling due to heavy concentrations of soot and smoke in the mid-troposphere. Mr Maddox seems to have missed the essential point repeatedly made by Turco *et al.* that smoke, because of its high absorption in the visible light range and low absorption in the infra-red, causes most of the surface cooling.

Thus the "Krakatoa effect" — namely,

a climatic and biological response to an eruption's aerosol veil — certainly supports the likelihood of a similar effect a nuclear war, but that the volcano-climate relationship alone is not wholly analogous to the far more complex and much more severe climatic and biological responses that Turco *et al.* (*Science* 222, 1283; 1983), Ehrlich *et al.* (*Science* 222, 1293; 1983) and Covey *et al.* (*Nature* 308, 21; 1984) have postulated. VALMORE C. LAMARCHE JR
*Laboratory of Tree Ring Research,
The University of Arizona,
Tucson, Arizona 85721, USA*

KATHERINE K. HIRSCHBOECK
*Department of Geography,
University of Oklahoma,
Norman, Oklahoma 73069, USA*

● The point of that part of the article complained of was to object that one of the principal references was then still "in preparation" and to assert that predictions of nuclear winter should be regarded as qualitative until more accurate data and calculations are available — Editor, *Nature*.

Lie detector lies

SIR — It is illuminating to express the results of studies in the fallibility of lie detection, such as that of Kleinmutz and Szucko¹, in terms of the amount of information obtained, which can be measured as the "expected weight of evidence" introduced by I.J. Good². Doing so reveals that what is obtained is a small fraction of the information required to take any rational decision.

Expressing experimental results as proportions: valid positive: proportion p of all actual liars, valid negative: proportion q' of all truth-tellers, false positive: $p' = 1 - q'$, false negative: $q = 1 - p$. The expected weight of evidence is the expected value of the logarithm of the Bayes' factor, which is

$$I = p \log(p/p') + q \log(q/q')$$

The logarithms can be taken to any base desired, and I follow Good² in using logarithms to base $10^{0.1}$ and calling the result "decibels" (db). Strictly, due allowance should be made for the small sample size, but for illustration I shall use the crude proportions as quoted by Kleinmutz and Szucko, which will tend to overestimate I . The value of I obtained for the results of their six interpreters are 1.3, 0.6, 2.8, 2.1, 0.8, 1.3, averaging 1.5 db (the variation is probably random rather than a reflection of varying skill). This is a very small amount of information. To make a confident decision in normal circumstances we usually need some 20 db — for instance, if we start with a hypothesis at "evens" (50 per cent probability), a log-factor of 20 db, that is a Bayes' factor of 100, will make it 100 to 1 on (99.01 per cent probability), which can perhaps be regarded as "practical certainty". In some pro-

posed applications of the lie detector, however, we are starting with an *a priori* improbable hypothesis — that an apparently honest person is a traitor, for instance. If the prior odds are estimated as 1,000 to 1 against, the log-factor required for practical certainty becomes 50 db.

If some procedure can be devised for collecting many independent scraps of information like this and combining them accurately, for instance by estimating credible log-factors and adding them, it might be possible to produce a reliable result. The "guilty knowledge test"³ which Kleinmutz and Szucko refer to is an attempt to do this: Lykken suggests using 10 to 16 independent items, which might add up to from 15 to 30 db. Where only the result of a single lie-detector test is available, the "confirmation" that it provides is so small that the only just course is to disregard it entirely. G.H. TOULMIN

30 Coltham Rd,
Cheltenham, Glos., UK

1. Kleinmutz, B. & Szucko, J.J. *Nature* 308, 449-450 (1984).
2. Good, I.J. *Probability and the Weighing of Evidence* (Griffin, London, 1950).
3. Lykken, D.T. *J. appl. Psychol.* 44, 258-262 (1960).

Crater dated

SIR — The cover of your 19 April issue shows an aerial photograph of Meteor Crater, Arizona. It is stated in the caption that the crater is the "result of an impact about 50 million years ago" but I am unaware of any Meteor Crater age estimates of this magnitude. The crater is clearly of Pleistocene age.

The age of Meteor Crater has been estimated using various lines of reasoning. It was observed 50 years ago that sediments at the crater show evidence of the Wisconsin glacial episode¹. These soils were later correlated with the well-studied stratigraphy at Hopi Buttes and it was observed that a Pleistocene sediment directly overlies the crater ejecta². Two major pluvial episodes of Pleistocene age are recorded as distinct erosional patterns within the crater³.

I have recently applied thermoluminescence (TL) dating to shock-metamorphosed sedimentary rocks from Meteor Crater (in preparation). Meteor Crater can be dated by the TL method because the TL of the target rocks was reset by the high temperatures experienced during the meteorite impact. After cooling, TL reaccumulated due to exposure to natural radioactivity. These measurements indicate that the Meteor Crater impact occurred 49,900 years ago with an estimated uncertainty of 2,900 years.

STEPHEN R. SUTTON
*McDonnell Center for the Space Sciences,
Washington University,
St Louis, Missouri 63130, USA*

1. Blackwelder, E. *Science* 76, 557-560 (1932).
2. Shoemaker, E.M. in *The Solar System* (eds Middlehurst & Kuiper) 301-336 (University of Chicago Press, 1965).
3. Shoemaker, E.M. & Kieffer, S.W. *Guidebook to the Geology of Meteor Crater, Arizona* (Center for Meteorite Studies, Tempe, Arizona 1979).

Tropical ecology research

SIR — The table below is compiled from the monthly listings of ecological publications in *Current Advances in Ecological Sciences* between 1979 and 1983. The journal (published by Pergamon, Oxford) cites an annual average of 15,000 recent ecological publications in about 2,000 source journals worldwide, under 56 major categories which I have condensed to 14 topics. The table shows a declining annual trend in tropical ecological research publications from 2.93 per cent of the global total for ecological publications in 1979 to about 1 per cent in 1983. The decline is also evident in each of the 14 areas listed except for tropical ecology (*sensu stricto*).

The major areas of research shown in the table are: (1) plant and animal interaction covering host/parasite relationships, competition and interaction of man and micro-organisms (24.2 per cent); (2) plant and animal communities — sociology, population dynamics and behaviour (14.0 per cent); and (3) growth, diseases and adaptation (11.3 per cent). Very little (0.2–2.4 per cent) tropical research work was done in (1) micrometeorology and the use of artificial environments for studying the functions of tropical plants and animals; (2) water relations and hydrology; and (3)

the ecology of various tropical plant communities as ecosystems.

The above trends indicate that tropical ecological research has been utilitarian, with emphasis on topics of direct benefit to mankind especially for food production. However, in view of the spread of desertification — which now affects 34 countries in Africa — and the economic crisis caused by the prolonged drought during 1982–83 in more than 24 African countries, including some coastal countries in the wet tropics, I think that much more emphasis should be placed on tropical ecological research dealing with water relations, hydrology and climate.

What is responsible for the decline in tropical ecological research publications? Can it be due to the effects of the economic recession and inflation on research work at universities and governmental institutions in developing countries in the tropics? One trend which has been obvious during the period is the massive migration of seasoned university staff and scientific research workers from the poorer developing countries in Africa to the more affluent developing countries in Africa as well as to the industrialized countries outside the continent. This brain-drain may have

contributed to the decline in ecological research work in the tropics; other factors may be the paucity of research funds and facilities, and of the trained middle level manpower needed to support field work.

N.H. AYODELE COLE

*Environment Section,
United Nations ECA,
Addis Ababa, Ethiopia*

Classic errors

SIR — R. H. Wright and R. E. D. Cattley (*Nature* 303, 568; 1983) and M. Macnamara (*Nature* 307, 590; 1984) discuss the possible meaning of Homer's "wine-dark" sea. Wright and Cattley propose a chemical answer to the problem, suggesting that wine when mixed with the alkaline water of the Peloponnese would turn blue. Macnamara, following W. B. Stanford's observation that the Mediterranean can look wine-red, assumes that the primary question is to account for the redness of the sea, and makes the extraordinary assertion that Homer "mentions only red wine in his epics".

Wright and Cattley have not faced up to the implications of the Ionian origins of Homer and much of Greek culture up to at least the seventh century BC. What happened in the Peloponnese may be irrelevant compared with what happened in Asia Minor.

And (*pace* Macnamara) wine in Homer can be black as well as red. At *Odyssey* 5.265 and 9.196 we meet the phrase *σκόρον... μέλανος οἴνου*, "a wine-skin of dark (black) wine". Thus "wine-dark sea" may mean a black sea no less than a red sea.

The Homeric adjective *οἴνωψ* (wine coloured) occurs in the variant forms *οἴωψ* in Sophocles and *οἴνωπος* in other Greek writers. At *Iliad* 18.562 we find *μέλανες... βότρυες*, ("a bunch of black grapes"); similarly, Sophocles (*Oedipus at Colonus* 674–5) applies *οἴνωψ* to ivy-berries. In fact wine was often described as "sparkling" in Homer (see *Iliad* 1.462 and 4.259), and the particular element of the sea which is important in the Homeric *οἴνωπα ποντον* is the sparkle of light reflected in the otherwise dark sea. Thus Theophrastus of Straton (*De Coloribus* 2.8) uses *οἴνωπος* to mean "black mixed with bright light". It may be (as Stanford believes) that *οἴνωπα ποντον* sometimes refers to a red sea. But this does not have to be the case, and to concentrate exclusively on the redness is to miss the real point. The sea is seen as a dark force, menacing and unpredictable (compare the primary meanings of the verb *πορφύρω* and the adjective *πορφύρεος*), and in this, as in wine, the light and the Sun are reflected (in the words of the poet) "in daggers of bright gold".

MARTIN PULBROOK

*Department of Ancient Classics,
St Patrick's College,
Maynooth, Co. Kildare, Ireland*

Trends in tropical ecological research 1979–83

	1979	1980	1981	1982	1983	Total (%)
General tropical ecology (historical, biogeography, dynamics of ecosystems)	43	38	16	16	10	123 (9.5)
Plant and animal communities	57	41	43	16	25	182 (14.0)
Productivity and energy	29	16	10	7	9	71 (5.5)
Plant/animal interactions	111	94	55	22	31	313 (24.2)
Biogeochemical cycles and nutrition	18	11	11	9	6	55 (4.2)
Water and hydrology	5	4	4	5	3	21 (1.6)
Growth, diseases and adaptation	51	45	26	14	10	146 (11.3)
Autecology, genecology and crop ecology	29	30	18	12	10	99 (7.6)
Micrometeorology and artificial environment	1	1	0	0	0	2 (0.2)
Soil biology, erosion, humus and microbes	22	20	5	7	6	60 (4.6)
Environmental problems (hazards, conservation, wildlife, pollution, weed and pest control)	26	22	26	13	21	108 (8.3)
Land use, surveys, remote sensing and statistical sampling	16	8	6	3	7	40 (3.1)
Tropical ecology	2	0	4	18	21	45 (3.5)
Forest, grasslands, freshwater and microbial ecology	5	9	11	5	1	31 (2.4)
Total entries for the tropics	415	339	235	147	160	1,296 100
% Of total entries (all subjects)	2.94	3.59	1.97	0.62	1.09	-

Yellow rain — a palynological analysis

Joan W. Nowicke* & Matthew Meselson*

Yellow rain, purported to be an agent of toxin warfare in South-East Asia, contains a high percentage of pollen. Analysis of this pollen in yellow rain samples suggests that they are the faeces of honey bees.

A HIGH pollen content has been reported in samples of yellow rain from South-East Asia¹⁻⁷. We have examined 11 samples of yellow rain allegedly collected at sites of chemical attack in Laos. The samples contain a high proportion of pollen. No two samples, even different spots of yellow rain on the same leaf, have the same composition of pollen types. The plant taxa identified are common in South-East Asia. Seven pollen types in yellow rain samples were also found in *Apis cerana* and honey from Thailand. In general appearance, high pollen content, and differences in pollen composition from one spot to another, the yellow rain samples resemble honey bee faeces.

The first two yellow rain samples we analysed were from small reddish-brown rocks with yellow spots. The third, the ABC News sample, is reported to have been scraped from vegetation by Hmong soldiers in Laos in March 1981 and to contain three trichothecene mycotoxins⁸. The remaining 8 samples were yellow spots from the upper surfaces of narrow lanceolate leaves about 15 cm long. The rocks and leaves were given to Canadian Embassy officials in Thailand in April 1982 by Hmong refugees who said they had collected them at two sites of chemical attack in Laos in March. The records do not specify whether the rock and leaf samples we examined are from one or both sites. All spots were 2–5 mm in diameter, agreeing with earlier descriptions^{1,2,7,9}. A 3 mm spot from a leaf weighed 1.5 mg and contained 1.1×10^5 pollen grains. We examined pollen from abdomens of two Asian honey bee species, *A. dorsata* and *A. cerana*, collected in Thailand by T.D. Seeley, who first suggested that yellow rain samples from South-East Asia might be faeces from cleansing flights of honey bees⁷. We also tested two samples of honey purchased in July 1983 at villages on the Thai-Lao border, and known faeces of *A. cerana* and *A. dorsata* on leaves collected in July and September, respectively, near Poona, India.

The first rock sample (Table 1, rock 1) was the residue on a piece of paper on which the rocks had been examined. This, and all other samples except as noted were acetolysed before scanning electron microscopy (SEM)¹⁰. It contained at least six pollen types. None could be confidently

identified even to family. The most numerous type, triaperturate and 20–23 µm long, is designated Fagaceae-like. The second sample, rock 2, was from a single spot. Its pollen consisted almost entirely of one of the minor types in rock 1, plus some grass pollen.

The ABC News sample consisted of four yellow fragments of approximately 2 mm³. SEM of an untreated fragment revealed vague outlines of pollen in an unknown matrix (Fig. 1a). Another untreated fragment, mounted in glycerine jelly for light microscopy, appeared to consist mainly of pollen, plus a few bee hairs (setae). The remainder of the sample was acetolysed, revealing about 20 pollen types, some of which are shown in Fig. 1b, d. Families identified were Compositae, Dilleniaceae, Elaeocarpaceae, Euphorbiaceae, Icacinaceae, Myrtaceae, Polygalaceae, and Sterculiaceae (Table 1). There was also a small proportion of grains possibly of Melastomataceae/Lythraceae. Grains were found that are indistinguishable from *Dillenia pentagyna* taken from herbarium specimens. Also found (Fig. 1d) were grains indistinguishable from those of *D. hookeri*, a species common in Laos¹¹. Another type in the ABC News sample (Fig. 1d) is indistinguishable from pollen from herbarium specimens of *Macaranga denticulata*, although its rather unspecialized morphology and the existence of some 300 species of *Macaranga*

make narrow identification difficult. The most common grain in the ABC News sample is very small, about 10 µm, with three apertures and a smooth/irregularly-pitted surface (Fig. 1d). It is classified as Elaeocarpus-like. Also, fungal hyphae and a few setae were seen in acetolysed preparations of the ABC News sample.

Leaf samples 1 and 2 were spots from different leaves, while 3A and 3B were spots a few cm apart on a single leaf, as were the sample pairs 4A/4B and 5A/5B (Table 1). Leaf samples 1 and 2 had three pollen types present in the ABC News sample — *M. denticulata*, Melastomataceae/Lythraceae and a pollen type tentatively placed in *Apodytes* (Icacinaeae). In addition there were hyphae, setae, and stellate, trichome-like structures. An abundant grain in leaf sample 1, probably from Moraceae/Urticaceae (Fig. 1e), was not seen in leaf sample 2. Conversely, an abundant grain in leaf sample 2, from *Ilex* (Fig. 1f), was not found in leaf sample 1.

Leaf sample 3A contained *M. denticulata*, *Ilex*, *Apodytes*, Melastomataceae/Lythraceae and a small proportion possibly of *Nuphar* (Nymphaeaceae). Sample 3B consisted mainly of Fagaceae-like grains, matching those in rock sample 1. The remaining leaf spots 4A, 4B, 5A and 5B, all had *Ilex*, *M. denticulata*, *Apodytes* and Melastomataceae/Lythraceae, but in very different propor-

Table 1 Summary of pollen identifications

	YELLOW RAIN											<i>A. cerana</i>	Honey 2
	Rock 1	Rock 2	ABC News	Leaf 1	Leaf 2	Leaf 3A	Leaf 3B	Leaf 4A	Leaf 4B	Leaf 5A	Leaf 5B		
AQUIFOLIACEAE <i>Dier</i>				++	+			*	*	*	*		
COMPOSITAE			*										
DILLENIACEAE <i>Dillenia pentagyna</i> <i>Dillenia hookeri</i>			+										*
EBENACEAE <i>Diospyros</i> -like												++	*
ELAEOCARPACEAE <i>Elaeocarpus</i> -like			++										+
EUPHORBACEAE <i>Macaranga denticulata</i>			+	++	++	++		+++	+	++	++		
FAGACEAE-LIKE	++						+++						*
GRAMINEAE		+										*	
ICACINACEAE <i>Apodytes</i>			*	++	++	+		*	+++	+	*		
MELASTOMATACEAE/ LYTHRACEAE			*	+	+	+		+	+	*	+		*
MORACEAE/URTICACEAE			++										
MYRTACEAE			*										*
POLYGALACEAE			*										
SAPINDACEAE													++
STERCULIACEAE <i>Sterculia</i>			*									+	
UNIDENTIFIED, 35–38µ 4 Apertures			+										*
UNIDENTIFIED, 25–35µ Punctate, 3-Colporate	+	+++											
UNIDENTIFIED, 45–50µ Thick Exine										+			

Key: *, minor component (<5%); +, 5–10% of sample; ++, 10–50%; +++, more than 50% of samples.

*Botany Department, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, USA (J.W.N.) and Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138 USA (M.M.)

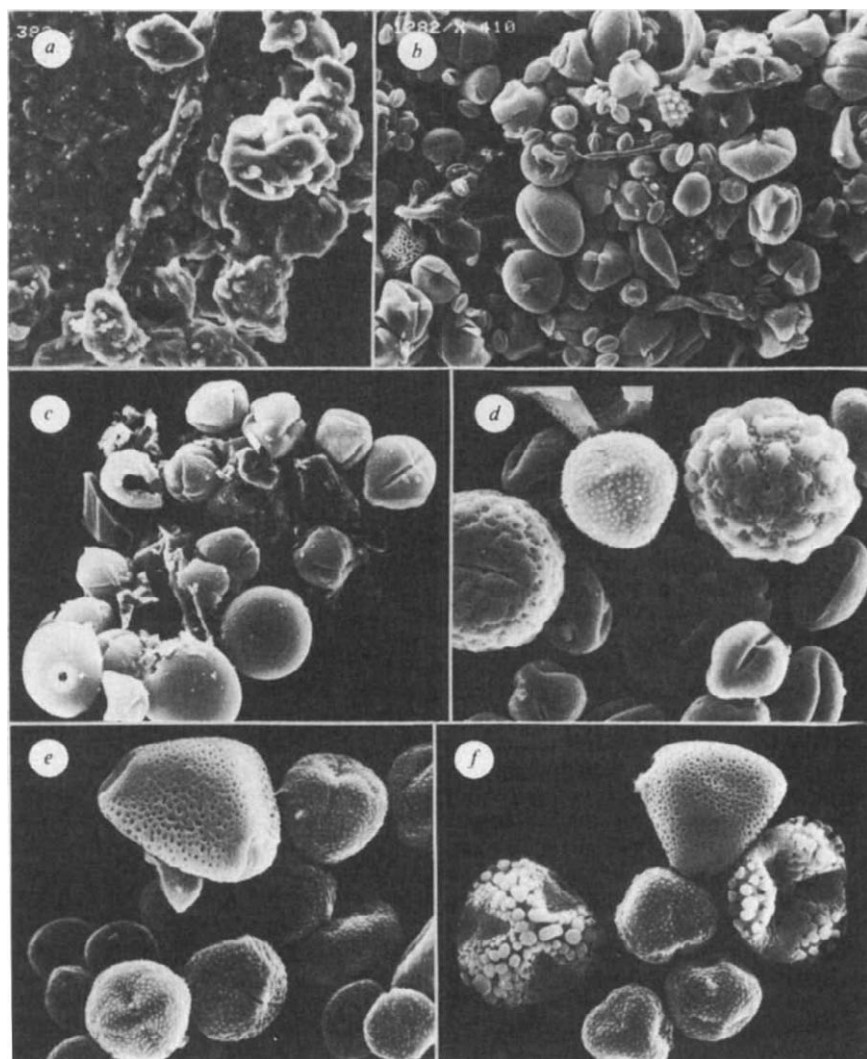


Fig. 1 SEMs of pollen in yellow rain. *a*, ABC news sample, untreated, X250. *b–f*, All acetolysed preparations. *b*, ABC News sample, X270. *c*, Rock sample 2, three spheroidal grains at bottom left are from the grass family, X330. *d*, ABC News sample, larger grain at upper right and partial grain middle left are probably *Dellenia hookeri*; grain between is *Macaranga denticulata*; the triangular grain in background is probably Myrtaceae; numerous smaller grains were classified as Elaeocarpus-like, X1,300. *e*, Leaf sample 1, the largest grain, seen in oblique view is assigned to *Apodytes*; the smallest grains, some showing pores, are Moraceae/Urticaceae; the remaining ones with furrows are *M. denticulata*, X1,170. *f*, Leaf sample 2, the uppermost triangular grain is *Apodytes*, the three small grains are *M. denticulata*, and the remaining two are *Ilex*, the one on the right is partially collapsed, X980.

tions in each case. In addition, 5A had an unidentified type 45–50 μm long, 3–4 aperture with a thick exine, that was not found in the other samples. The great diversity of the ABC News sample as compared with the leaf and rock samples may be a consequence of collection technique — a number of yellow spots may have been combined.

An abdomen of *Apis cerana* collected in March 1980 contained at least three pollen types: Diospyros-like, a small proportion of grains from the grass family (Gramineae) and a reticulate type 35 μm long, matching in form and range of variation a type in the ABC News sample tentatively assigned to *Sterculia*. *A.*

dorsata, collected in March 1980, had pollen probably representing two or three closely related species in *Castanopsis/Lithocarpus* (Fagaceae).

Honey sample 1, from Nakhon Phanom (17° 25'N; 104° 45'E) had at least ten pollen types, but none matched those in the yellow rain samples. Honey sample 2, from Chiang Khan (17° 55'N; 101° 45'E) contained many pollen types including Fagaceae-like, seen in rock sample 1 and leaf sample 3B; the type designated Melastomataceae/Lythraceae, found in the ABC News sample and in leaf samples 1, 2, 3A, 4A, 4B, 5A and 5B; and four more types seen in the ABC sample: *D. pentagyna*,

Elaeocarpus-like, and an unidentified four-aperturate grain, 35–38 μm long.

Known faeces of *A. cerana* and *A. dorsata* on leaves collected near Poona, India resembled spots of yellow rain in size, general appearance, high pollen content and the presence of setae and hyphae. Each of three spots of *A. cerana* faeces had at least five pollen types, with only some common to all three. Faeces of *A. dorsata* from a nearby site showed similar diversity from spot to spot.

Our identifications of pollen in yellow rain samples were compared with those in a report from the Australian Defence Department². One of their three samples had mainly Harpullia-like pollen (Sapindaceae); the other two were predominantly Rapanea-like (Myrsinaceae). A Chinese study describes a "yellow rain" phenomenon in September 1976 in Jiangsu¹². Occurrences generally lasted a few minutes, covering areas of 0.5–20 acres with 2–6mm yellow spots rich in pollen. The authors concluded that the spots were honey bee faeces. Mass cleansing flights of *A. mellifera*, causing similar phenomena, have been studied previously^{13,14}.

All of the plant taxa we identified in samples of yellow rain are common in South-East Asia. The three assignments we made at the species level, *D. hookeri*, *D. pentagyna* and *M. denticulata*, are trees native only to Southern Asia. Also, a reticulate type in the ABC sample assigned to *Sterculia* was present in *A. cerana* collected in the same season in Thailand. Most significantly, honey sample 2 contained six pollen morphotypes we found in yellow rain, showing that these pollen types are gathered by honey bees in Indochina. No two samples of yellow rain we examined had the same pollen composition; even adjacent spots on the same leaf were different. Pollen diversity from spot to spot was also found for known bee faeces from India. Such differences between spots would not be expected if the spots of yellow rain had been disseminated by an artificial source. Our observations lead us to conclude that the yellow rain samples we examined are probably honey bee faeces.

We thank Diane Baldwin for preparation of the manuscript, Janice Bittner for preparing pollen samples, Joseph Rosen for the ABC News sample, the Government of Canada for rock and leaf samples, George Eickwort for museum specimens of Asian honey bees, Amos Townsend and Dennis Meseroll for honey samples, Fred Dyer for samples of bee faeces from India, Peter Ashton for helpful discussions and Janet Peterson for technical assistance. □

Received 12 December 1983; accepted 20 March 1984.

1. Sukroongreung, S., Kritalugsana, S., Nilakul, C., Thakerngpol, K., and Viriyanonndha, P. *Siriraj Hospital Gazette* 34, 643–647 (1982).
2. Crone, H.D. *Tech Report* OCD 82/14 (Australian Defence Scientific Service, August 1982).
3. Crone, H.D. & Tantar, V. *Tech. Report* OCD 83/13 (Australian Defence Scientific Service, August 1983).
4. U.S. Department of State, *Briefing on Chemical Warfare*, p.19–20 (29 Nov. 1982); p.11–13 (30 Nov. 1982).

5. UN General Assembly, document A/37/259 (1 December, 1982).
6. Marshall, E. *Science* 221, 242 (1983).
7. Ashton, P.S., Meselson, M., Robinson, J., Perry P. & Seeley, T.D. *Science* 222, 366; 368 (1983).
8. Rosen, R.T. & Rosen, J.D. *Biomed Mass Spectrometry* 9, 443–450 (1982).
9. Hearing before the Subcommittee on Asian and Pacific Affairs, Committee on Foreign Affairs, US House of Representatives, 12 December 1979, page 26.

10. Dickison, W.C., Nowicke, J.W. & Skvarla, J.J. *Am. J. Bot.* 69, 1055–1073 (1982).
11. Vidal J. *La Végétation du Laos. Travaux du Laboratoire Forestier de Toulouse* Tome V, Vol. I, Article III: 1–582 (1960).
12. Chang Chung-Ying, Chen Yu-ming, Chou Shu & Li Min. *Kexue Tongbao* 22, 409–412 (1977).
13. Nitschmann, J. *Deutsche Entomologische Zeitschrift* 4, 143–171 (1957).
14. Riemann, G. *Bienenzucht* 11, 121 (1958).

Cell biology

Epidermal growth factor receptor—yet another domain?

from Shelley Earp

CELLS responsive to epidermal growth factor (EGF) carry specific receptors for EGF on their surface membrane. Between 12 and 24 hours after EGF binds to these receptors, the cells begin to synthesize DNA. In the meantime, the majority of the EGF-receptor complexes have been removed from the cell surface by a process of internalization into cellular vesicles^{1,2}. A long-standing question has been whether the cellular events triggered by EGF are solely a consequence of intracellular messages generated at the cell surface by ligand-receptor binding or whether the internalized complex can have additional intracellular functions. A radical re-examination of this question will be forced by the paper on page 270 of this issue of *Nature* in which Mroczkowski, Mosig and Cohen suggest that the EGF receptor has a direct effect on the structure of DNA³.

The suggestion stems from the authors' detection of an endonuclease activity in preparations of EGF receptor highly purified by affinity chromatography. The activity is specific for the nicking of double-stranded circular DNA and is stimulated by ATP but not by a non-hydrolysable ATP analogue. In those respects it resembles the enzyme DNA topoisomerase II but it has not been possible to demonstrate for the EGF receptor the DNA circle-closing activity characteristic of topoisomerase II⁴.

The authors' interest in the latter enzyme, which catalyses topological rearrangements of DNA that are thought to be important in the regulation of transcription and replication⁵, may stem from several reports that relate EGF receptor and topoisomerases. First, EGF increases DNA topoisomerase II activity in human foreskin fibroblasts⁶ and this activity is also increased during liver regeneration⁷, when EGF receptor internalization is known to occur⁸. Second, Wang and colleagues have shown that topoisomerases are covalently bound to the 5' end of nicked DNA through a phosphotyrosine linkage⁹; the EGF receptor is well known to have tyrosine-specific protein kinase activity and to autophosphorylate its own tyrosine residues. While it is difficult to envisage the same catalytic site both transferring phosphate and forming protein-DNA linkages, the autophosphorylation site on the receptor could also serve as a topoisomerase-binding site.

As the authors point out, an important unresolved question is whether the endonuclease activity of the receptor preparation is the result of a contaminating enzyme detected by a highly sensitive assay.

The concentration of receptor used is higher than would be necessary to detect receptor autophosphorylation. Most assays were conducted with 4–16 molecules of receptor per DNA circle and the limit of detection of endonuclease activity was 0.4 molecules per circle. The poor catalytic activity could be due to loss of activity during purification but the nuclease seems to be more stable towards heat denaturation than the receptor's tyrosine-specific protein kinase activity.

The dissociation of nuclease and kinase activity is troubling in another sense. Mroczkowski *et al.* indicate that purified pp60^{src}, the product of the transforming gene of Rous sarcoma virus, has an endonuclease activity similar to that of the EGF receptor. Both pp60^{src} and the EGF receptor exhibit tyrosine-specific kinase activity. If the endonuclease and kinase activities can be dissociated one might expect to see two distinct regions of sequence similarity between pp60^{src} and the EGF receptor. And yet the two proteins seem only to be related by sequence in the kinase domain¹⁰.

Another nucleotide-binding site on the receptor would also need to be invoked to explain the differences in nucleotide specificity of the kinase and endonuclease activities of the EGF receptor—for example, GTP can be used by the former but not latter¹¹. The nucleotide specificity of the reported endonuclease activity also differs from that of topoisomerases which, at equivalent concentrations of enzyme and DNA, can be stimulated by non-hydrolysable analogues of ATP¹².

Given there is little doubt that receptor occupancy causes immediate alterations in membrane function, probably related to stimulation of the receptor's protein kinase acting through tyrosine phosphorylation of perimembrane substrates, autophosphorylation of aggregated EGF receptors, or, as recently proposed, through phosphorylation of phosphatidylinositol^{13,14}, does the endonuclease activity of the EGF receptor have biological relevance? If it has, receptor internalization must be a prerequisite for EGF action. After a decade of research, the necessity of internalization for the mitogenic response of cells to EGF has not been established—but neither has it been ruled out. So it is worth considering several potentially relevant points about the consequences of internalization.

Since the EGF receptor has been shown to be proteolytically processed upon internalization¹⁵, one possibility is that the process yields a new structure that dictates its own translocation to a specific site of action, such as the nucleus. If the translocated fragment acts catalytically only a small portion of internalized receptor would need to be segregated to the nucleus to produce a biological effect. It may also be of relevance that proteolysis seems to increase the activity and alter the topological specificity of certain nucleases¹⁶. Perhaps the EGF receptor is a pronuclease whose activity and catalytic repertoire can be increased by proteolysis. If proteolytic activation simultaneously eliminated the binding and antigenic domains of the receptor that are external to the cell surface, the fact that neither EGF binding nor receptor immunofluorescence has been consistently observed in the nucleus could be explained. If the catalytic endonuclease moiety generated by proteolysis had a short half life, it would account for the need to terminate the function it served after an appropriately short time. Any hypothesis that EGF releases a latent endonuclease activity must take into account the fact that EGF does not cause growth in all receptor-bearing cells, that EGF binding and action are influenced by other humoral agents and that in some cells the need for EGF can be abrogated by combinations of other factors^{17–20}. It is possible that an endonuclease domain could be processed differentially depending on the cell type or the influence of modulating factors, but would cells opt for another level of complexity in their growth control network?

Several questions raised by the provocative findings of Mroczkowski *et al.* should quickly be resolved. Can the EGF receptor form a phosphotyrosine linkage with DNA? Can conditions be found to express the topoisomerase II-like circle-closing activity? Does experimental proteolytic cleavage of the receptor produce a receptor fragment with greater or more definable endonuclease activity? Is the ATP-stimulated DNA-nicking an intrinsic activity of the EGF receptor or will

1. Carpenter, G. & Cohen, S. A. *Rev. Biochem.* **48**, 193 (1979).
2. Adamson, E.D. & Rees, A. R. *Molec. cell. Biochem.* **34**, 129 (1981).
3. Mroczkowski, B., Mosig, G. & Cohen, S. *Nature* **309**, 270 (1984).
4. Gellert, M. A. *Rev. Biochem.* **50**, 879 (1981).
5. Lilly, D.M.J. *Nature* **305**, 276 (1983).
6. Miskimins, R. *et al. Expt Cell Res.* **146**, 53 (1983).
7. Duguet M. *et al. Nucleic Acids Res.* **11**, 1059 (1983).
8. Rubin, R.A., O'Keefe, E.J. & Earp, H.S. *Proc. natn. Acad. Sci. U.S.A.* **79**, 776 (1982).
9. Tse, Y.-C., Kirkford, K. & Wang, J.C. *J. biol. Chem.* **225**, 5560 (1980).
10. Downward, J. *et al. Nature* **304**, 521 (1984).
11. Carpenter, G., King, L. & Cohen S. *J. biol. Chem.* **254**, 4887 (1979).
12. Sugino, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 4838 (1978).
13. Sugimoto, Y. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 2117 (1984).
14. Macara, I.G., *et al. Proc. natn. Acad. Sci. U.S.A.* (in the press).
15. Das, M. & Fox, C.F. *Proc. natn. Acad. Sci. U.S.A.* **175**, 2664 (1978).
16. Chow, T. Y.-K. & Fraser, M.J. *Can. J. Biochem.* **57**, 889 (1979).
17. Mondschein, J.S. & Schomberg, D.W. *Science* **211**, 1179 (1981).
18. Westermark, B. *et al. in Growth and Maturation Factors* Vol. I (ed. Guroff, G.) 73 (Wiley, New York, 1983).
19. Rozenfurt, E. *Molec. biol. Med.* **1**, 169 (1983).
20. Wharton, W. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 5567 (1982).

a contaminating nuclease be separated from receptor preparations? Cohen has been consistently linked with new advances in the field of growth factors. His pioneering efforts in identifying and purifying EGF and its receptor have led to the discoveries that have set the rest of us in new directions. The new data from his laboratory direct attention to the nuclear

effects of EGF and will undoubtedly stimulate discussion and experimentation. However before clamouring to exchange acrylamide for agarose, cell biologists should await further developments. □

Shelley Earp is a member of the Cancer Research Center and Department of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514.

Retinitis pigmentosa

Progress in sight

from Miranda Robertson

THERE is no doubt that molecular genetic technology is leading to an understanding of human genetic disease at a depth and resolution inaccessible to more traditional methods. Because the path to that understanding is often indirect, however, for the time being it presents a problem for clinicians as well as a challenge for research scientists. This is inevitable when the gene defect underlying the disease is unknown, and can be approached only through linked genetic markers. Such is the case in Duchenne muscular dystrophy, for which a marker was first reported two years ago¹, and Huntington's disease, for which a marker was discovered last year². On page 253 of this issue of *Nature*, Bhattacharya, Wright and their colleagues report an exactly similar marker for the X-linked form of retinitis pigmentosa³.

As with Duchenne and Huntington's, the retinitis pigmentosa marker is simply an identifiable fragment of DNA that is close enough to the gene to be inherited with it, putting molecular biologists within 'walking' distance of the defective gene itself and offering clinicians the prospect of identifying some symptomless carriers of the gene and, possibly, diagnosing their unborn babies. What are the obstacles confronting the research biologists and the problems facing the clinicians?

Retinitis pigmentosa is the name given to a genetically and phenotypically heterogeneous group of degenerative diseases of the photoreceptor cells of the retina leading, in severe cases, to blindness. The mode of inheritance can be recessive, autosomal dominant or X-linked. The X-linked form, for which Bhattacharya *et al.* now have a marker, is one of the most severe; it accounts for perhaps 30 per cent of all cases and affects about 1 in 20,000 of the population. Because the defective gene is on the X chromosome, of which males have only one, they have no normal gene to compensate for its effects and are severely affected, often in childhood. Females, with two X chromosomes, may have a mild form of the disease or none at all; but they risk transmitting the severe form to their sons. Female carriers of the gene, and the daughters of known carriers, who have no way of knowing whether they themselves are carriers, stand to gain most from the

new marker. But it will not help them all, for reasons that follow from the nature of linked markers in general.

The marker in this case is a DNA probe designated L1.28, corresponding to a small fragment of the human X chromosome. What allows this fragment to be used as a marker is its polymorphism — which is to say that it contains minor variations that can be detected by bacterial restriction enzymes (for a slightly fuller explanation of how this works, see ref. 4). L1.28 has two distinguishable variants, A1 and A2. Bhattacharya *et al.* were able to establish the linkage of L1.28 with the retinitis pigmentosa gene on the X chromosome by tracing the inheritance of both the disease and the A1 and A2 variants through five different families.

In the case of close linkage, you would expect the disease to be inherited consistently with one or other of the two variants within a given family; and this is what Bhattacharya *et al.* found. In four families retinitis pigmentosa was consistently inherited with A2; in the other one it was associated with A1. It is just possible that more than one gene on the X chromosome can give rise to retinitis pigmentosa. If so, the linkage will break down in further family studies. So for the time being clinical advice will not be offered on the basis of the L1.28 marker.

But assuming, as seems most likely, that there is only one gene, what are the limitations of L1.28? Most obviously, an affected male relative must be available to establish whether the defective gene is associated with A1 or A2 in the family in question. The second important limitation arises from the fact that both A1 and A2 are present at high frequency in the normal population. Take the case of a young woman whose father has the disease. Since one of her two X chromosomes must be her father's, and his single X chromosome has the defective gene, she will be an obligate carrier and there is an even chance that any son born to her will inherit retinitis pigmentosa. As things stand, she would be offered termination of any pregnancy in which the fetus was male — the entire X chromosome serving as a crude and unreliable marker for the disease. The L1.28 marker makes it possible to distinguish

fetuses bearing the normal X chromosome of the mother from those bearing the defective one — but only if the mother is heterozygous for the marker: that is, if she has A1 on one of her X chromosomes and A2 on the other. In that case, if her father's X chromosome bears the A1 variant she can be certain that any male fetus inheriting A1 will also inherit retinitis pigmentosa, and conversely if he inherits A2 he will be normal. But if she is homozygous — that is, she has A1 on both X chromosomes — there is no way of distinguishing a normal male from an affected one before birth. Given the frequency of the two variants in the general population, only 40 per cent of women will be heterozygous.

Similar considerations complicate the case of a woman for whom the family history of retinitis pigmentosa is on her mother's side. Because the symptoms of the disease in women develop late or never, she may not know even whether she is a carrier. If her mother is homozygous for the marker, the marker cannot enable her to find out; though it may, if she herself is heterozygous, enable the disease to be ruled out in some of her unborn sons.

Finally, even for a heterozygote, there is always the slight chance (decreasing with the closeness of the linkage) of misdiagnosis if the defective gene has become separated from the linked marker by genetic recombination. The identification of a second linked marker, on the other side of the gene, would dramatically reduce the probability of mistaken diagnosis; it would also be helpful in the pursuit of the retinitis pigmentosa gene itself.

This, of course, is the longer-term aim of the research: in the end the only entirely reliable genetic probe is the one that identifies the mutant gene. Progress will demand patience and ingenuity. Patience because the distance between L1.28 and the retinitis pigmentosa gene is something on the order of 3 million bases; so to reach the immediate vicinity of the gene will require either a marathon chromosome walk or laborious family studies to find more closely linked markers; and ingenuity to identify the gene once it has been isolated. For retinitis pigmentosa, the best bet may be to compare the genes expressed in normal retinas with those expressed in affected retinas, by means of cDNA libraries from the two tissues.

How soon might a gene probe for X-linked retinitis pigmentosa be available? Should female carriers or suspected carriers wait a year or two before starting families in the hope of reliable prenatal diagnosis? In the case of Duchenne muscular dystrophy, there is still no gene probe two years after the first marker; but optimists expect one this year. □

1. Murray, J.M. *et al.* *Nature* **300**, 69 (1982).
2. Gusella, J.F. *et al.* *Nature* **306**, 234 (1984).
3. Bhattacharya, S.S. *et al.* *Nature* **309**, 253 (1984).
4. Robertson, M. *Nature* **306**, 222 (1983).

Miranda Robertson is Acting Life Science Editor of Nature.

Oceanology

Storms in the abyss

from P.F. Friend

ANY lingering notion that the dark waters of the deep ocean floors are uniformly calm should finally be dispelled by the vivid account of this remarkable environment provided by Hollister and McCave starting on pages 220 of this issue of *Nature*.

Their study concentrates on a 2×4 km area of the Atlantic Ocean, where it reaches a depth of almost 5 km, some 500 km south of Halifax, Nova Scotia. Although the surface waters there move generally eastwards, as part of the Gulf Stream, the deepest waters tend to move westwards as part of a distinctive deeper western boundary current that is cold (less than 1.9°C) and perhaps has its origins in the bottom water of the Antarctic. This cold bottom current has a remarkably high velocity of up to 0.73 m s^{-1} , similar to those measured in strongly moving surface tidal flows or in flooding rivers.

Another striking feature of the area is that it is subject to 'storm' periods of several days to a few weeks in which bottom currents intermittently reach speeds of over 0.3 m s^{-1} and are often reversed. Calculations of the variance of current speed provide a measure of the so-called eddy kinetic energy of the system. It seems that high values of the eddy kinetic energy in deep waters are usually found in areas where the eddy kinetic energy at the surface is also high, as in the Gulf Stream. The kinetic behaviour of the deep currents in storms is perhaps best visualized as an intermittent and reversing surge; there is no sign of the oscillatory periods of several seconds that are typical of surface-storm kinetics.

Because the muddy ocean floor in the area studied is rich in organic nutrients, it supports many actively burrowing organisms that soon disrupt any sedimentary features. The fact that the top layer of the floor retains sedimentary features therefore indicates that it was recently and rapidly deposited. The concentration of sediment can be as high as 12 g m^{-3} in these deep-bottom waters, similar to that in some muddy estuaries. These concentrations, coupled with the major variations in the small structures of the ocean bottom recorded in time-series of photographs, show that the vigorous movements of the bottom currents erode and transport large amounts of the fine-grained sediment. Indeed, careful examination of sediment cores from the ocean bed reveals the rapid rates of deposition of sediment and the occurrence of frequent episodes of erosion: several centimetres of mud may be deposited in the course of a few days and then removed equally rapidly. By such a process more than 3,000 times more material may have been deposited in

the 10,000 years of the Holocene than is apparent from the deposition rate of 5 cm kyr^{-1} recorded in the sediments, according to the estimates of Hollister and McCave. It is common to find that the rates of short-term sedimentation (and erosion) are several orders of magnitude higher than those of long-term accumulation for the shallow-sea deposits in many old sedimentary basins but it has not hitherto been known that the same is true on the floors of deep oceans.

Considering all the deep oceans of the

Earth, Hollister and McCave point to seven discrete areas, most of them tens to hundreds of kilometres across, where the suspended load of the deep-ocean water is particularly high. All the areas occur below zones of high surface-eddy kinetic energy, and probably contain deep currents which move strongly and reverse intermittently. These few, rather small areas may be the prime sources of much of the suspended muddy material of the world's oceans just as a few land areas, which have erodible soils and intermittently strong winds, seem to contribute a large part of the very fine-grained suspended terrigenous materials in the Earth's atmosphere. □

P.F. Friend is in the Department of Earth Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EQ.

Colour vision

Direct recordings from the cone cells of a primate

from J. D. Mollon

FACED with the infinite varieties of hues and tints in a sunlit wood in spring, it can be difficult to believe that all our sensations of colour arise from just three unidimensional retinal signals. Nevertheless, our ability to see in colour depends on no more than a comparison of the relative rates of absorption of photons in the three types of cone cell within each local region of the retina and on the distribution of these ratios across the retina. The three types of cone cell contain photopigments with peak sensitivity in different parts of the visible spectrum; although direct measurements of their absorbance spectra have been possible for many years, Nunn, Schnapf and Baylor have achieved a great technical feat in obtaining the first direct electrophysiological recordings from a primate cone cell, reported on page 264 of this issue¹.

The existence of three types of retinal receptor was first suggested 200 years ago^{2,3} and by 1886 the brilliant Arthur König had accurately deduced the spectral sensitivities of the cones from psychophysical studies of normal and colour-blind observers⁴. But the three types of cone remained, in effect, hypothetical constructs until the first direct measurements of the absorbance spectra of the different cone types were obtained by microspectrophotometry, in which a narrow monochromatic measuring beam is passed through the outer segment of an individual receptor cell. Nowadays relatively precise estimates of the three absorbance spectra are available for monkey and human retinas^{5,6}.

An absorbance spectrum — a plot of the optical density of the cell at different wavelengths — is not, however, necessarily

the same as an electrophysiological action spectrum, which shows the number of photons required at different wavelengths to produce a given electrical signal. For example, it might have been the case that the electrical signal produced by an absorbed photon was dependent on wavelength. Moreover, the steep long-wavelength limbs of the absorbance spectra can be followed only down to values around one-tenth of the maximum absorbance, whereas action spectra are not so limited and it is easy to measure the intense lights needed to produce a criterion electrical signal at long wavelengths.

It is physiological action spectra that Nunn, Schnapf and Baylor have now provided us with. To measure them in the tiny (perhaps $2 \mu\text{m}$ by $10 \mu\text{m}$) pigment-containing outer segments of primate cones, they adapted a clever trick that Yau, Lamb and Baylor⁷ introduced in 1977 originally to make recordings from the much larger photoreceptors of the toad. Instead of trying to put a microelectrode inside the outer segment, Yau *et al.* put the outer segment inside a micropipette, sucking it gently in and allowing the mouth of the pipette to form an electrical seal round the base of the outer segment. The signal thereafter measured is a graded reduction in the current that flows continuously into the outer segment in the dark.

In their samples of tissue from macaque monkeys, Nunn *et al.* have been able to record only middle- and long-wavelength cones; the peak sensitivities of the two types of record lie quite close together in the green (540 nm) and yellow-green (570 nm) regions of the spectrum, in good correspondence with both psychophysical

and microspectrophotometric estimates. The short-wave receptors, the so-called 'blue cones', have so far eluded measurement by the new method. But this is not surprising, for psychophysical, microspectrophotometric and histological results all converge to suggest that the blue cones are relatively sparse, perhaps accounting for less than 10 per cent of all cones in human and macaque retinas. It seems that we use the blue cones only for colour vision *per se*. For the detection and resolution of contours, for stereopsis and for discrimination of movement, we rely on visual subsystems that draw their inputs only from the long and middle-wave cones⁸.

Of particular interest to many psychophysicists will be Nunn *et al.*'s observation that the electrical responses of primate cones have a shorter time course than do those of rods from the same species. Such a difference has long been postulated in order to explain why our ability to resolve rapid flicker is poorer, and why the subjective duration of our sensations is greater, when our vision depends on responses of the rods rather than of the cones⁹.

Building on this pioneering work of Nunn, Schnapf and Baylor, it will be possible to answer some interesting questions. First, there is the question of adaptation. Nunn and Baylor have already reported that primate rods exhibit little intrinsic gain control as the illumination level is increased¹⁰. Will this also be the case for primate cones? Second, will there prove to be a residual discrepancy between absorbance and action spectra for primate cones and, in particular, is the quantum efficiency the same for the short-wave 'β band' as for the primary mode of the spectral sensitivity curve? These questions will be securely answered only when microspectrophotometric and electrophysiological measurements are made concurrently on the same primate cones. Third, will there prove to be individual differences in the exact spectral positions of cone sensitivities as has been suggested by microspectrophotometric data from human eyes⁶? □

1. Nunn, B.J., Schnapf, J.L. & Baylor, D.A. *Nature* **309**, 264 (1984).
2. Palmer, G. *Theory of Colours and Vision* (Leacraft, London, 1777).
3. Palmer, G. *Théorie de la Lumière, Applicable aux Arts* (Hardouin & Gattey, Paris, 1786).
4. König, A. *Report of the 56th meeting of the British Association*, 431 (Murray, London, 1886).
5. MacNichol, E.F., Levine, J.S., Mansfield, R.J.W., Lipetz, L.E. & Collins, B.A. in *Colour Vision* (eds Mollon, J.D. & Sharpe, L.T.) (Academic, London, 1983).
6. Dartnall, H.J.A., Bowmaker, J.K. & Mollon, J.D. *Proc. R. Soc. B220*, 111 (1983).
7. Yau, K.-W., Lamb, T.D. & Baylor, D.A. *Nature* **269**, 78 (1977).
8. Mollon, J.D. *Doc. Ophthal. Proc. Ser.* **33**, 87 (1982).
9. Hecht, S. & Verrijp, C.D. *Proc. natn. Acad. Sci. U.S.A.* **19**, 522 (1933).
10. Nunn, B.J. & Baylor, D.A. in *Colour Vision* (eds Mollon, J.D. & Sharpe, L.T.) (Academic, London, 1983).

J. D. Mollon is in the Psychological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EB.

Molecular biology

Anatomy of hypersensitive sites

from Sarah C. R. Elgin

In the analysis of the structure of chromatin in animal cells, sites hypersensitive to digestion by the enzyme DNase I are generally found within the 1,000 base pairs (bp) that flank the 5' ends of genes that are active or can be activated (see ref. 1 for a brief review). That these discontinuities in the chromatin fibre mark sites relevant to the control of gene expression has been shown in several instances, most directly in an analysis of deletion mutations at the *Drosophila Sgs4* glue protein locus², and they can be considered as sites of potential interaction between specific effector proteins and DNA. A critical question is that of how the DNase I hypersensitive sites are generated and maintained; answers should not be long delayed to judge by the progress that has already been reported this year or is soon to be published.

The appearance of DNase I hypersensitive sites precedes the initiation of transcription and is tissue-specific for genes so regulated. Studies of the mini-chromosome of simian virus 40 (SV40) and of the chick β-globin gene indicate that DNase I hypersensitive sites correspond to regions of DNA that are nucleosome free³⁻⁵. Since it is known that DNA in certain conformations is unlikely to form stable nucleosomes, a formal possibility is that the DNase I hypersensitive sites consist solely of a short stretch of DNA in one such conformation. In that respect there has been considerable recent interest in sites that are sensitive to S₁ and other 'single-strand-specific' endonucleases in regions 5' to genes in supercoiled DNA and in chromatin^{6,7}. Some are sensitive to S₁ nuclease but not *Bal31* nuclease, while others show the opposite pattern¹¹. The sites frequently correspond to short stretches of double-stranded DNA where one strand is all purines and the other all pyrimidines⁸⁻¹⁰. The exact form of the altered structure at these sites in the supercoiled DNA is unknown but it appears unlikely that truly single-stranded regions are formed^{10,11}.

While the available data are sketchy, it is common to observe, 5' to genes, one of several classes of sequence capable of adopting some sort of altered conformation. Comparisons of DNA mapping data with chromatin mapping data indicate that in at least some cases, such sequences flank DNase I hypersensitive sites of chromatin instead of being an integral part of them (for example, refs 11,12). This is not to suggest that they are necessarily without influence; data both from artificial rearrangements of the SV40 genome and from *Drosophila* variants at the *Sgs4* locus indicate that sequences adjacent, or within a few hundred base pairs, can be critical for

the formation of DNase I hypersensitive sites^{2,13}. A model in which alternative DNA structures play a part in generating DNase I hypersensitive sites is attractive^{6,7}. One's enthusiasm must, however, be tempered by the fact that there is relatively little evidence of whether alternative forms of DNA actually occur *in vivo*.

It seems likely that DNase I hypersensitive sites are generated by the interaction of nonhistone proteins with the DNA, perhaps following cues from the surrounding sequence, perhaps binding to the region itself. The challenge to isolate such proteins has recently been taken up by Emerson and Felsenfeld¹⁴. Using as an assay the correct assembly of the DNase I site 5' to the chick β -globin gene, they have separated from the nuclei of chick erythrocytes, in which the gene is expressed, a protein that not only produces the requisite nuclease sensitivity but also retains a DNA fragment containing the 5'-flanking region in a filter-binding assay. Binding experiments with different DNA fragments set one boundary of the recognition site between 268 and 303 bp, and the other between 40 and 164 bp, 5' of the transcription start site. For comparison, the DNase I hypersensitive site maps between 65 and 163 bp, micrococcal nuclease sensitivity extends to 260 bp (ref. 5) and a tract of 16 consecutive deoxyguanosine residues, sensitive to S₁ nuclease in the supercoiled form, occupies bp 178-197 (refs 9 and 10). The region of interest therefore covers both the DNase I hypersensitive site in chromatin and the most prominent S₁ nuclease sensitive site in the DNA, but not the 'TATA box' or the transcription start site. Obviously more than one critical protein-DNA interaction may be occurring.

Fortunately, several new techniques have recently been developed which improve our ability to map or 'footprint' both alterations in DNA structure and protein-DNA interactions. In a forthcoming issue of *Nature*, M.M. Becker and J.C. Wang will describe a 'photofootprinting' technique, which uses ultraviolet light to detect protein-DNA contacts as well as changes in the structure of DNA at the sequence level. The technique is based on the observation that the photochemical reactivities of DNA bases are dependent on both protein-binding and DNA structure. Breakage of the DNA backbone only at the sites of photodamage and end-labelling by any of several techniques allows a map of relative photoreactivity to be developed. The question of which conformations of DNA at 5' sites with potential alternative structures are present *in vivo* might profitably be addressed by this method in

the immediate future.

And on page 229 of this issue, Wu introduces a new approach to analysing the position of DNA-binding proteins within or around hypersensitive regions¹⁵. He uses exonuclease III of *Escherichia coli* to digest chromatin within the hypersensitive site after its cleavage by a suitable endonuclease (DNase I or a restriction enzyme). The exonuclease is thought to stop when it encounters a protein, thus defining one boundary of the binding site. In an analysis of a heat-shock protein gene (*hsp70*) of *Drosophila*, a resistant (protein-bound) site is found 12–40 bp 5' of the transcription start site in normal cells; in heat-shocked cells, however, an additional resistant site is observed in the region between 40 and 108 bp.

Techniques recently devised to cross-link protein with DNA should provide a second method for mapping these interactions, and the potential to identify the proteins involved, although at present this is frequently limited to cases where antibodies against the protein are available

(for example, refs 16,17). Concerted application of these techniques could lead to a full definition of the DNase I hypersensitive site in molecular terms within the next few years. □

1. Elgin, S.C.R. *Cell* **27**, 413 (1981).
2. Shermoen, A.W. & Beckendorf, S.K. *Cell* **29**, 601 (1982).
3. Jakobovits, E.B. *et al.* *Nature* **285**, 263 (1980).
4. Saragosti, S., Moyne, G. & Yaniv, M. *Cell* **20**, 65 (1980).
5. McGhee, J.D. *et al.* *Cell* **27**, 45 (1981).
6. Larsen, A. & Weintraub, H. *Cell* **29**, 609 (1982).
7. Weintraub, H. *Cell* **32**, 1191 (1983).
8. Mace, H.A.F., Pelham, H.R.B. & Travers, A.A. *Nature* **304**, 555 (1983).
9. Nickol, J.M. & Felsenfeld, G. *Cell* **35**, 467 (1983).
10. Schon, E. *et al.* *Cell* **35**, 837 (1983).
11. Selleck, S.B., Elgin, S.C.R. & Cartwright, I.L. *J. molec. Biol.* (in the press).
12. Nordheim, A. & Rich, A. *Nature* **303**, 674 (1983).
13. Jongstra, J. *et al.* *Nature* **307**, 708 (1984).
14. Emerson, B.M. & Felsenfeld, G. *Proc. natn. Acad. Sci. U.S.A.* **81**, 95 (1984).
15. Wu, C. *Nature* **309**, 229 (1984).
16. Karpov, V.L., Preobrazhenskaya, G.V. & Mirazbekov, A.D. *Cell* **36**, 423 (1984).
17. Gilmour, D.S. & Lis, J.T. *Proc. natn. Acad. Sci. U.S.A.* (in the press).

Sarah C.R. Elgin is in the Department of Biology, Washington University, St Louis, Missouri 63130.

Archaeological dating

New method for bricks?

from E. T. Hall

AN unexpected byproduct of investigations of the chemical stability of clays employed in the storage of nuclear waste is the suggestion of a new method of dating bricks, ceramics and other materials fired in antiquity. The technique, suggested by Waddell and Fountain¹, depends on the diffusion of Ca²⁺ ions from mortar or cement layers into the clay brick. The authors report a relationship between time and calcium content at the interface of the clay brick with the mortar but base their report on only seven samples. These show that there is an increase of calcium with time, although the experimental nature of the curve relating the distance migrated by calcium to time is far from clear. The migration distances are small (< 20 µm in 200 years), whereas the matrix of bricks is comparatively coarse. An electron microprobe was used for the analyses, providing a resolution of 2.5 µm; there can be little doubt that the analytical results from the traverses across the cement-brick interface must be very 'noisy' and accurate measurement difficult to achieve, even though multiple passes were made to obtain an average result.

The new technique is interesting but still at a preliminary stage of development and its application to real archaeological dating may well be problematical. Clearly it has a long way to go before it can rival the most successful of the methods for dating fired materials — thermoluminescence. The work of Aitken^{2,3} and others⁴ has made thermoluminescence the standard procedure⁵ for testing the authenticity of doubtful ceramics, particularly when

public or private purchasers are contemplating their purchase in the saleroom. For bricks whose environment is known, so that the thermoluminescent component arising from their surroundings or from cosmic rays can be estimated, thermoluminescence can be expected to provide dating that is accurate to within ~7 per cent.

Another technique for dating bricks and other fired clays is archaeomagnetic dating⁶, where the past history of the Earth's magnetic field, both in direction and intensity, is used. In the case of bricks, which will have the direction of the Earth's field induced in them at the time of firing, we do not normally know the orientation of the brick when fired and so measurement of direction of the brick's magnetic moment will not help us. However, recent measurements⁷ of intensities of magnetization, which can be related to the ancient field strength, have provided some evidence that this might become a useful method of dating, albeit at a lower accuracy than is provided by thermoluminescence. □

1. Waddell, C. & Fountain, J.C. *Geology* **12**, 24 (1984).
2. Aitken, M.J., Zimmerman, D.W. & Fleming, S.J. *Nature* **219**, 442 (1968).
3. Aitken, M.J. *Phys. Rep.* **40C**, 277 (1978).
4. Mejdahl, V. *Archaeometry* **14**(2), 245 (1972).
5. Fleming, S.J. *Med. Sci. Law* **14**(1), 11 (1982).
6. Weaver, G.H. *Archaeometry* **9**, 174 (1966).
7. Aitken, M.J., Alcock, P.A., Bussell, G.D. & Shaw, C.J. in *Geomagnetism of Baked Clays and Recent Sediments* (eds Creer, K.M. *et al.*) 122 (Elsevier, Amsterdam, 1983).

E. T. Hall is Professor in the Research Laboratory for Archaeology and the History of Art, University of Oxford, 6 Keeble Rd., Oxford OX1 3QJ.

Fractals

Aggregationism

THE problem of aggregation is both ancient, as in the manufacture of carbon black, and modern, with the recognition in the past few years that microscopic structures as different as snowflakes and colloidal precipitates may be examples of physical structures with fractional dimensions — fractals in the sense intended by Hausdorff in 1919 and more recently given wide currency by the proselytizing of B.B. Mandelbrot. One page 225 of this issue R.M. Brady and R.C. Ball report one of the most direct demonstrations so far that aggregation does yield fractal structures.

Brady and Ball are concerned only with aggregation whose rate is limited by that at which extra particles can reach the surface of a growing cluster. The circumstances therefore do not necessarily correspond to those which determine the growth of, say, a snowflake from water vapour, where the shape of the crystal may be governed by the rate at which latent heat can escape. Either way, the application of macroscopic arguments to the growth of these structures is hampered by the non-linear character of the instabilities that form on growing surfaces (departures from regular shape grow more quickly) whence the interest in the microscopic simulation introduced by T.A. Witten and L.M. Sander three years ago (*Phys. Rev. Lett.* **47**, 1400; 1981).

That model is simple enough. Put a seed particle at the centre of a finite patch of, say, a square lattice and allow fresh particles to enter the system at random places on the periphery and to make their way through the lattice by random walking, following the rule that they stick to the growing aggregate whenever they reach a neighbouring position on the lattice. Brady and Ball confirm the computer prediction for the three-dimensional lattice (that the density should be a decreasing function of the radius of an aggregate) by their neat set-up in which the electrolysis current at constant voltage is simultaneously both a measure of distance and, when integrated over time, a measure of the total mass.

As it happens, an experimental investigation of the other obvious case of aggregation — that typified by the aggregation of colloidal particles in homogeneous suspension — has also just been published (Weitz, D.A. and Oliveria, M. *Phys. Rev. Lett.* **52**, 1433; 1984). The model, in this case, is one in which the particles initially occupy many sites of a lattice, then diffuse randomly through it and stick together whenever they encounter another particle or growing aggregate. The unpublished result of the simulation (due to P. Meakin) is said to be a fractal dimension of about 1.75, which Weitz and Oliveria say they have confirmed by the measurement of individual particles of colloidal gold in aggregates formed by controlled flocculation. □

Role of RecA protein spiral filaments in genetic recombination

Paul Howard-Flanders*, Stephen C. West* & Andrzej Stasiak†

* Department of Molecular Biophysics and Biochemistry, and Department of Therapeutic Radiology, Yale University, Box 6666 New Haven, Connecticut 06511, USA and † Institute for Cell Biology, ETH-Honggerberg, 8093 Zurich, Switzerland

Physical and enzymatic studies on RecA protein from Escherichia coli provide the basis for a molecular model of general genetic recombination, a novel feature of which is the role attributed to spiral filaments of RecA protein.

In general genetic recombination, homologous chromosomes are paired, cut and joined to form chromosomes with new linkages. To examine the underlying mechanisms, data from genetic crosses in fungi, bacteria and viruses have been used to develop models that account for the genetic data. More recent biochemical and physical studies on RecA protein, the recombination enzyme of *Escherichia coli*, allow the development of more complete molecular models for the pairing and exchange reactions involved. In this article we describe such a molecular model, introducing it with a brief review of published models and the studies on which it is based.

Models of genetic recombination

Several alternatives have been suggested to explain how homologous pairing between chromosomes is initiated: (1) when both duplexes are nicked by an enzyme at identical positions in each chromosome¹; (2) when one duplex carries a single strand gap and the other is nicked^{2,3} (Fig. 1a); (3) when one duplex is nicked and a strand is displaced by local DNA synthesis⁴ (Fig. 1b); (4) when both duplexes are replicated locally⁵; (5) when particular sites in DNA are recognized by initiation enzymes⁶; (6) when a two-strand break is made (Fig. 1c)⁷; or (7) when one duplex carries a single-strand gap and the other is intact⁸⁻¹¹ (Fig. 1d). The more recent models were designed to form unequal lengths of heteroduplex DNA, as required by the data on meiotic recombination in fungi^{7,12,13}. However, it has proved difficult to determine from genetic data how recombination is initiated, and the choice between models remains open.

Homologous recombination also serves a function in the repair of certain types of DNA damage. In yeast, for example, the repair of double-strand breaks depends on recombination with a homologue¹⁴. Similarly, the switch between mating types is attributed to gene conversion at the mating type locus, initiated by a double-strand break¹⁵. In bacteria, recombination is initiated by DNA containing incised cross-links¹⁶ or by post-replication gaps which induce sister exchanges with an efficiency close to unity⁸.

In *E. coli*, both general genetic recombination and post-replication repair require *recA*⁺, a pleiotropic gene that is activated by DNA damage^{17,18}. The *recA*⁺ gene product¹⁹⁻²³ is a protein of 38,000 molecular weight (M_r) and known sequence^{24,25} that polymerizes into spiral filaments. It has a single-strand DNA-dependent protease activity for SOS induction in response to DNA damage²³, and it mediates enzymatic reactions with DNA of relevance to recombination³.

DNA binding sites

In the presence of the non-hydrolysable ATP analogue, ATP(γ -S), RecA protein tends to aggregate at low pH into long filaments²⁶, and at neutral pH it condenses on duplex DNA in rod-like filaments²⁷. On careful examination of shadowed speci-

mens in an electron microscope, the filaments formed on DNA are seen to be striated and to consist of right-handed spirals of pitch 100 Å and diameter roughly 100 Å (Fig. 2a, b). When negatively stained, the spiral filaments appear to be deeply grooved with space between adjacent turns. They occur singly or in bundles in quasicrystalline alignment and appear hexagonal in cross-section²⁸.

The number of turns of spiral filament needed to cover a circular plasmid of defined length is very reproducible, indicating that RecA binds stoichiometrically to duplex DNA. There is one turn per 18.6 base pairs (bp) and there are 6.2 RecA monomers per turn, as determined by counting RecA striations on plasmid DNA and by measurements of the linear density of the filaments^{29,30}. Evidently the spiral protein filament is wound along the phosphodiester backbone of the duplex with each monomer binding to exactly the same number of base pairs^{28,29,31}. This number may be 3 bp, as assumed in Fig. 4, but not all methods give the same value³². Measurements of overall length of these filaments show the DNA to be stretched by 50% beyond the normal B-DNA length. At 18.6, instead of the usual 10 bp per turn, the duplex is underwound by nearly 50%, the angular twist between adjacent base pairs being reduced from the normal value of 36° to about 20° (refs 28, 29).

RecA protein filaments also form on circular single-strand DNA in the presence of ATP, but are neither stretched nor striated. Some complexes are seen by electron microscopy to be of similar length to B-DNA, but others are collapsed on themselves. They are unstable and few are seen unless single-strand binding protein is present, or they are fixed with glutaraldehyde³³.

Certain features of RecA spiral filaments have been revealed by X-ray crystallographic methods. RecA protein crystallizes in hexagonal and in tetragonal crystals, which have been examined by X-ray diffraction³⁴. The hexagonal crystals which form at low pH appear to contain spiral chains with 6 RecA protein monomers per turn of length 84 Å and symmetry P6₁ (Fig. 2c). Since the spiral filaments seen by microscopy are right handed, we think the monomers may also be arranged in right-handed spirals within the crystals. The X-ray diffraction studies on hexagonal crystals prove that the spiral filaments consist of RecA monomers arranged head to tail in equivalent positions (Fig. 2c), and not in a sequence of dimers or tetramers³⁴. The head-to-tail orientation of monomers provides a physical basis for the functional directionality of the enzyme in strand exchange reactions³⁵⁻³⁷. This directionality can be understood if each monomer has two DNA binding sites, one for each DNA molecule to be paired. In contrast there would be no basis for directionality if each monomer had only one DNA binding site and the functional unit was a symmetrical dimer.

In summary, these physical methods show that RecA protein binds to DNA stoichiometrically and forms right-handed spiral

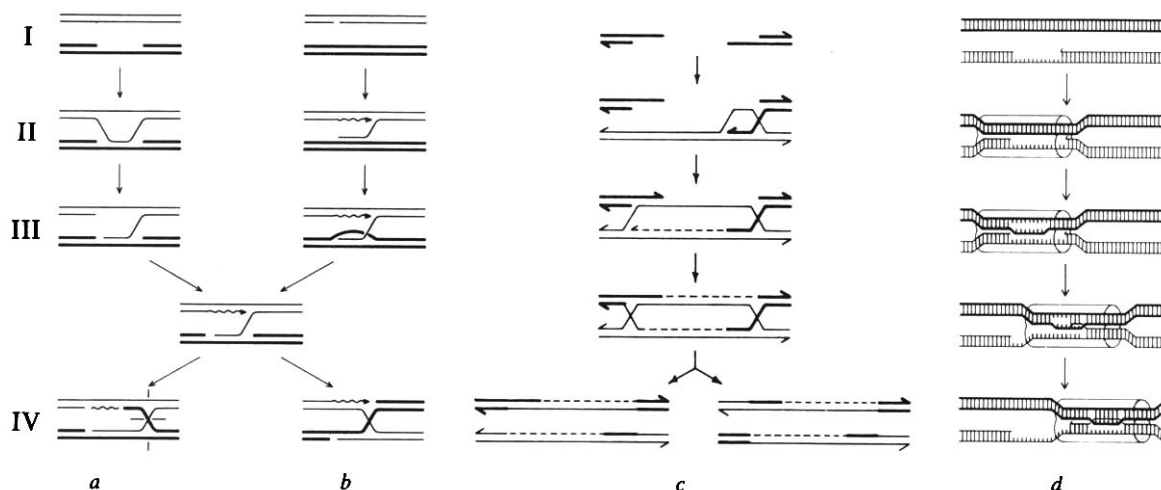


Fig. 1 Current models for the mechanism of general genetic recombination. *a*, (I) Recombination initiated by a single-stranded gap. (II) Unwinding and pairing. (III) Paired strand is cut. (IV) Repair synthesis. (V) Isomerization and resolution of one crossover to form recombinants^{2,3,10}. *b*, (I) Recombination initiated by a nick. (II) DNA synthesis at nick displaces strand. (III) Homologous pairing. (IV) Intact duplex is cut in response to pairing. (V) Isomerization and resolution of one crossover to form recombinants⁴. *c*, (I) Recombination initiated by a single-strand gap. (II) pairing by first 3' end. (III) Repair synthesis from 3' end, second 3' end pairs. (IV) Repair synthesis from second 3' end completes gap repair and formation of two cross-overs. (V), (VI) Resolution of two cross-overs to form recombinants⁷. *d*, (I) Recombination initiated by a single-strand gap. (II) RecA protein condenses on gap and promotes homologous pairing. (III) Strand exchange to form heteroduplex at gap. (IV) Branch migration to the right allows end of strand to interwind with duplex. (V) Strand crosses back. The gap is repaired and the two cross-overs are resolved to form recombinants. The intact homologue is cut only at the resolution of the cross-overs¹¹.

filaments of regular structure with all the monomers orientated in the same direction.

Initiation, pairing and strand exchange

RecA protein binds cooperatively to single-strand DNA and has a single-strand DNA-dependent ATPase activity^{38,39}. Homologous pairing by RecA protein was first detected *in vitro* when single-strand fragments were paired with circular duplex DNA to form stable complexes⁴⁰⁻⁴³ referred to as displacement loops or D-loops. However, the pairing and strand exchange reactions of this protein have been more fully characterized in reactions with circular single-strand and full length linear duplex DNA^{44,45}. RecA protein binds to single-strand DNA to form an initiation complex that is capable of immediate pairing with duplex DNA^{46,47}. One strand of the linear duplex is transferred to the single-strand circle, to form a nicked circular heteroduplex and a linear single strand⁴⁴. The strand exchange requires ATP and advances in the 5' to 3' direction along the original single strand at a rate of only a few bases per second^{35,36}. It works

best if single-strand binding protein (SSB) is added to stabilize the initiation complex, and has the advantage that the substrates, intermediates and products of the reaction can all be assayed.

Homologous pairing and strand exchange reactions also occur between two duplex molecules if one of them contains a small single-stranded gap (gapped DNA)^{9,10}. When gapped circular DNA is incubated with RecA protein and linear duplex DNA containing a 3' end complementary to the gap, RecA pairs the molecules starting in the gap^{48,49}. Strand exchange then proceeds in the 5' to 3' direction in the gap and continues along the adjoining duplex to form two heteroduplexes^{11,37}. When the gapped DNA is incubated with nicked circular DNA, pairing at the gap leads to the 3' ends being exchanged and to the formation of figure-8 DNA structures containing Holliday cross-overs⁵⁰.

The reaction between gapped and intact circular duplex DNA is similar. Again, homologous pairing initiated at the single-strand gap, leads to strand exchange and the formation of heteroduplex DNA, but in this situation the length of heterodu-

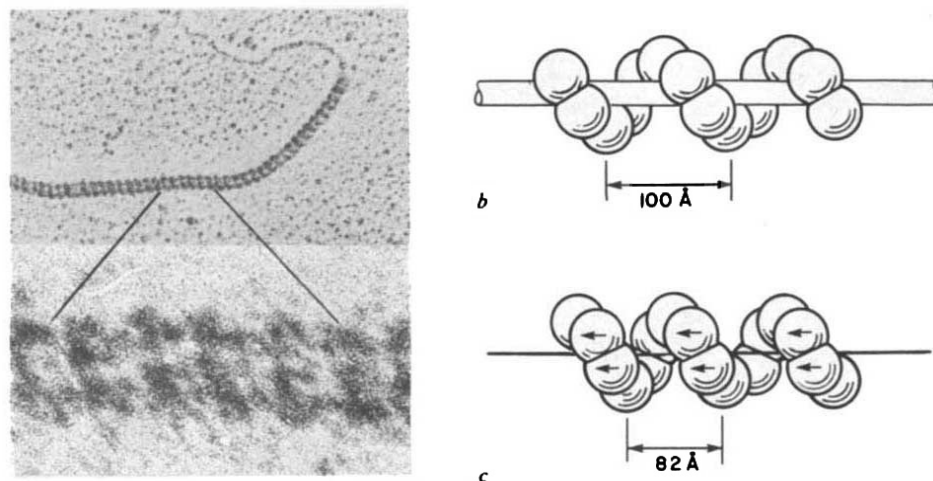


Fig. 2 Structures of RecA protein spiral filaments. *a*, RecA protein spiral filament formed on circular duplex DNA in the presence of ATP(γ -S), shadowed with Pt and seen by electron microscopy²⁸. *b*, Diagram of RecA bound to duplex DNA in the presence of ATP(γ -S), as determined by electron microscopy²⁸. RecA monomers are shown as spheres, but their exact shape is unknown. *c*, Diagram of RecA spiral filament in crystals of RecA protein free of DNA, based on X-ray crystallographic data. Arrows indicate alignment of monomers³⁴.

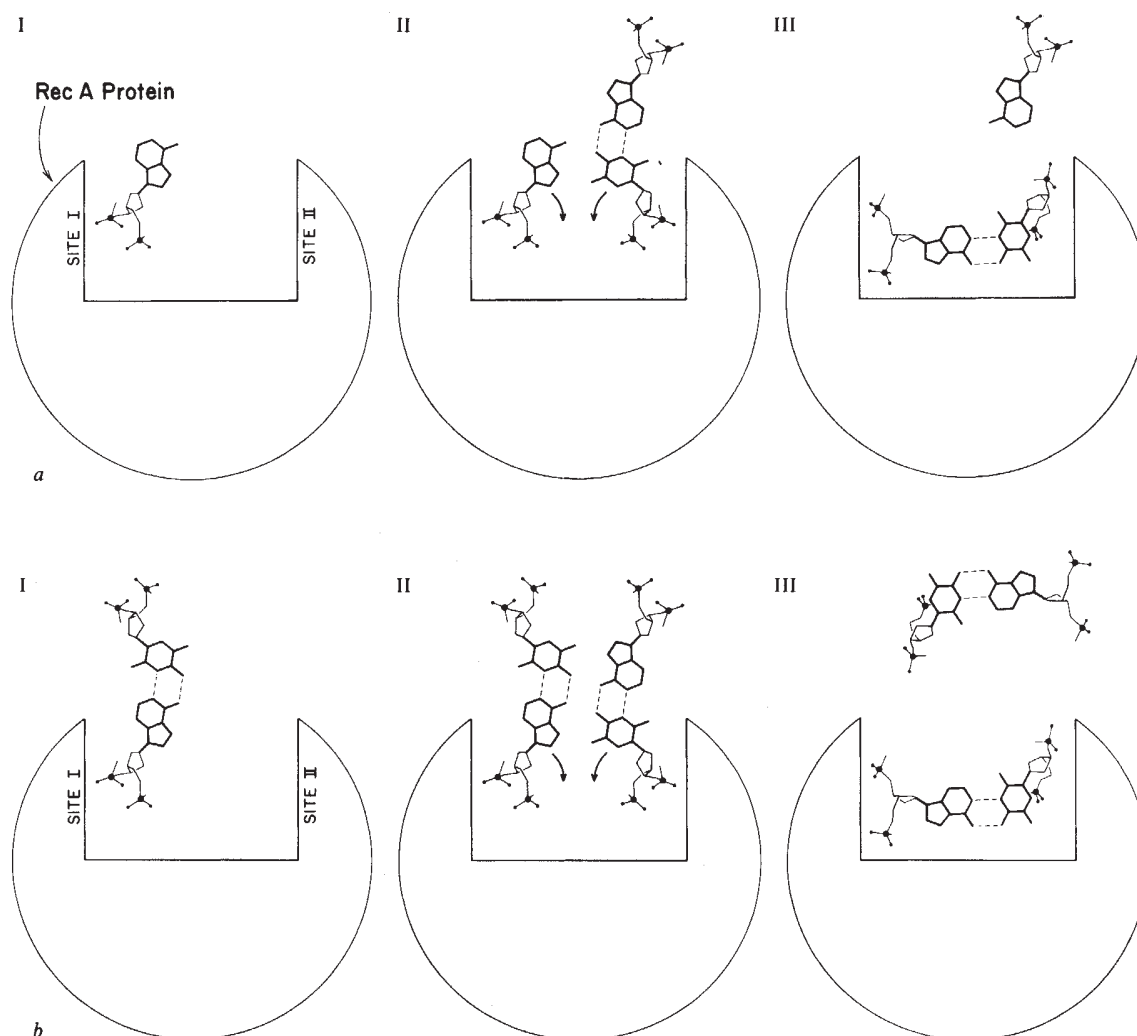


Fig. 3 A two DNA binding site model for RecA protein and a proposed mechanism for homologous pairing and strand exchange. Each base is shown in axial view with two phosphates. The sugar phosphates are unwound to about 20° between adjacent base pairs. RecA protein is about 50 Å in diameter, 60% larger than shown in this diagram. *a*, Pairing and strand exchange between single strand and duplex. (I) A single-strand binds at site I of RecA protein to form an initiation complex. (II) Initiation complex binds to one strand of a duplex at site II with homologous pairing by hydrogen bonding (bonds not shown) in the wide groove. (III) RecA protein rotates the bases into the heteroduplex position. *b*, Pairing and strand exchange between two duplexes. (I) One strand of the duplex binds at site I to form a duplex initiation complex. (II) The duplex initiation complex binds to the second duplex with homologous pairing by hydrogen bonding (bonds not shown) in the wide groove. (III) RecA protein rotates the bases into the heteroduplex configuration.

plex is limited by topological constraints and an interwound figure-8 carrying two Holliday cross-overs is formed^{10,11}.

Search for homologous contacts

Several mechanisms for homologous pairing can be visualized: (1) RecA protein may move two DNA molecules relative to each other until homologous sequences come into alignment, or (2) pairing may result from random contacts between DNA molecules. In support of the latter mechanism, RecA pairs single-stranded and duplex molecules if they share a common base sequence of 100 bp or more, but fails to do so if homology is limited to 30 bp. The rate of homologous pairing is directly proportional to the length of the DNA molecule, even if partially non-homologous⁵¹. Moreover, RecA protein forms aggregates with DNA, that are readily seen by electron microscopy and must locally increase the concentration of both naked and complexed DNA^{27,33}. These results are consistent with a pairing mechanism in which DNA, aggregated by RecA protein, makes an increased number of random contacts at multiple points along chains. Such contacts will of course be transient unless homologous sequences touch and pairing is established.

The alignment of identical base sequences at homologous pairing presumably depends upon sequence specific complementary hydrogen bonding between DNA chains. Two forms can be visualized: (1) The two strands of a duplex may be separated and able to pair as Watson-Crick heteroduplexes. This form of pairing may occur in the absence of RecA when superhelical circular phage DNA is annealed with homologous single-strand fragments to form D-loops⁵². However, it is not at all clear that this form of pairing occurs in RecA-mediated reactions, particularly since *in vitro* tests for RecA-mediated strand separation of duplex DNA in advance of pairing have given negative results⁵³⁻⁵⁵; (2) Initiation complexes may pair in the wide groove of the duplex. Complementary base pairing between a single-strand and a fully base-paired duplex could depend on hydrogen bonds between the oxygen and amino groups at positions 4 on pyrimidines and positions 5 and 7 on purines. A four-stranded structure that depends on such bonding has been proposed⁵⁶⁻⁵⁸. Poly(dT) · poly(dA) · poly(dT) is not of exactly the same structure, but is an example of a stable three-stranded deoxyribose polynucleotide chain⁵⁹.

Although we cannot rule out either form of pairing on the

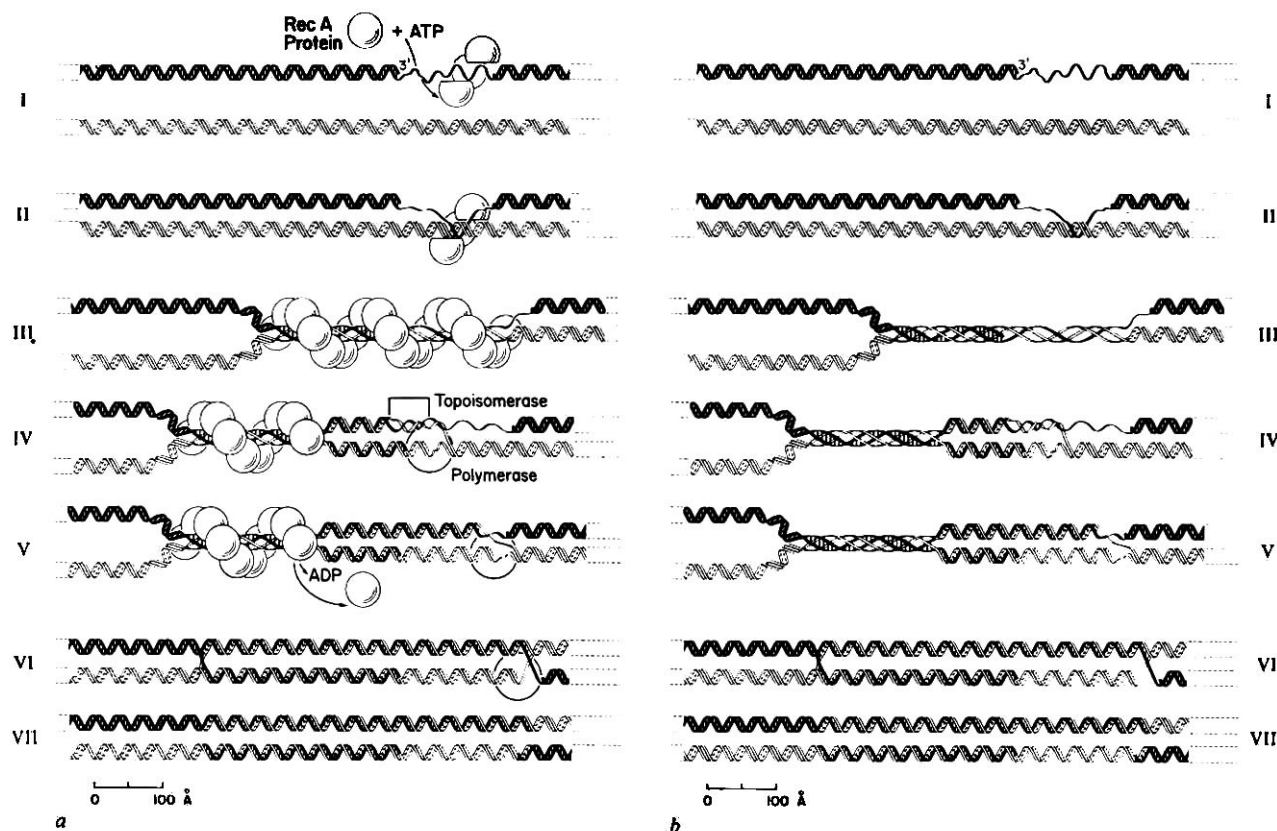


Fig. 4 An enzymatic model for genetic recombination. *a*, Complete model with enzymes; *b*, DNA only. (I) RecA protein binds cooperatively to the single strand in a gapped duplex to form an initiation complex, in preparation for pairing. Watson-Crick base pairs are shown as rods linking the DNA twin helices. (II) The initiation complex binds to the intact duplex, making transient contacts until a homologous site is reached. For clarity, the initiation complex is drawn with only a few protein monomers, but in reality is likely to extend over hundreds or thousands of nucleotides. (III) When homologous contacts are made and the strands become paired locally, the initiation complex acts as nucleus for further cooperative binding, which extends the RecA spiral filament around all three paired strands. As more RecA binds, the spiral filament extends around both duplexes drawing them together, wide groove facing wide groove. RecA binds to duplex molecules in response to pairing. (IV) The spiral filament is extended further by the addition of more monomers to the head end. RecA monomers at the tail end switch the strands to the heteroduplex configuration and may then be released from the DNA. The upper heteroduplex is relaxed by topoisomerase I. DNA polymerase starts to repair single strand gap by adding nucleotides to 3' end (broken lines). (V) RecA driven branch migration continues with hydrolysis of ATP. (VI) Growth of the spiral filament ceases and the last RecA monomer dissociates, leaving single strand cross-overs, which are then resolved enzymatically. The crossover on right is generated by switching the base stacking (isomerization). (VII) Mismatched base pairs in the heteroduplexes are corrected enzymatically and mature recombinants are formed.

basis of experimental data, we think wide groove pairing to be more likely because of the failure to detect strand separation before pairing and because wide groove contact would hold the strands in position for the ensuing strand exchange reaction. Moreover, it is valid for pairing between single-stranded and duplex DNA as well as for the pairing between two duplexes that should occur in reactions between gapped and intact duplex DNA⁹⁻¹¹ (see also Fig. 3*a*(II), *b*(II)). Although stable four-stranded polydeoxyribonucleotide chains have not been found, they may be formed when two duplexes are held together within a spiral filament. However, the duplexes probably would not be in the normal B-form, nor in a fully paired four-stranded structure with 3.4 Å axial spacing as has been proposed^{56,57}. Since RecA spiral filaments on duplex DNA are regular structures in which the DNA is underwound and stretched by 50% (refs 28, 29), the four-stranded structure is likely to be regular rather than a mixture of left- and right-handed turns, and to be symmetrical about the helical axis.

Surprisingly, strand exchange reactions do not require the substrate to be undamaged or even that the duplexes are fully homologous. For example, in a three-strand reaction, strand exchange is affected very little by deletions of 10 or 20 bp^{60,61} or by pyrimidine dimers⁶². Similarly, deletions of a few base pairs in one duplex do not block strand exchanges between two duplexes⁵⁰. Thus, in contrast to the initiation of homologous pairing which is sensitive to departures from homology, the

strand exchange reaction advances along each duplex with little regard for small deletions or base damage.

RecA binding to duplex DNA

The binding of RecA protein to DNA molecules exhibits several unexpected features. In the presence of ATP, it normally binds only transiently to covalent circular duplexes. However, the addition of homologous single-strand DNA fragments results in a more stable form of binding of RecA to the double-stranded DNA, which then becomes temporarily immune to subsequent pairing with single-strand fragments⁶³⁻⁶⁵. This immunity may be due to the RecA protein which, starting from the paired initiation complex, binds cooperatively to the circular duplex and causes local underwinding. The remainder of the form I DNA becomes overwound and the whole molecule becomes immune to pairing. Further evidence for underwinding by RecA is obtained in similar reactions in the presence of topoisomerase I. Again the addition of homologous single strands leads to extensive RecA binding and to a marked reduction in DNA winding number^{54,64}.

In summary, RecA binds to ssDNA to form initiation complexes which are ready for pairing, but it binds to duplex DNA in response to homologous pairing, forming a complex which is immune. These results provide additional evidence that each RecA monomer has two DNA binding sites (Fig. 3*a*). Initiation complexes formed by interaction of the ssDNA fragment with

site I, pair with duplex DNA which binds at site II. Since the resulting ternary complex acts as a nucleus for further cooperative RecA binding beyond the terminus of the single stranded fragment, the duplex DNA becomes covered by RecA protein and is subsequently immune to further pairing.

A molecular model for recombination

Based on the information discussed above, we have constructed a molecular model for general genetic recombination in *E. coli*. It differs from previous models by proposing that RecA protein forms a spiral filament that surrounds the paired DNA molecules. The strand exchange reaction takes place within the spiral filament and switches base pairing to form two heteroduplexes. To illustrate this mechanism, we show a diagram of an exchange between two duplexes initiated at a single-strand gap, but anticipate that this mechanism may be applicable to recombination initiated in other ways.

The assumptions made in developing this model are as follows: (1) Each RecA monomer has two distinct DNA binding sites, the primary site and the secondary site (Fig. 3a(I) and (II)). (2) In an initiation complex, the primary binding site in each RecA monomer is filled by single-strand DNA. (3) Pairing is between an initiation complex and a naked duplex. (4) The RecA spiral filament winds round the paired DNA molecules and dissociates after strand exchange.

Figure 4a shows an outline of the proposed molecular model for recombination between two duplexes, and Fig. 4b shows the same model with enzymes removed, so the DNA strands can be seen more clearly. In this scheme, genetic recombination is initiated by the duplex containing a single-strand gap. Since RecA protein forms initiation complexes on single-strand DNA⁴⁶, we will assume it also does so on gapped DNA (Fig. 4aI), binding initially to the single-stranded DNA in the gap and subsequently along the adjoining duplex²⁷. Once formed, initiation complexes pair with naked duplex DNA (Fig. 4aII). For clarity, the initiation complex is drawn in Fig. 4 with only a few protein monomers, but in reality is likely to be many times longer.

Homologous pairing between gapped and intact duplex molecules may occur when the initiation complex (Fig. 4aI) with single-stranded DNA at site I (Fig. 3aI), makes homologous contacts with a naked duplex, winding around all three strands (Fig. 4aII) with the duplex at site II (Fig. 3aII). Compensating left-hand turns which must form outside the paired regions, are not shown⁵⁸. The paired initiation complex may serve as a nucleus for further cooperative binding, extending the spiral filament to the end of the gap and along the adjoining duplex, drawing both duplexes together (Fig. 4aIII). In a similar way, when site I is filled by duplex DNA, it behaves as an initiation complex and is again capable of interacting with a naked duplex which binds in site II (Fig. 3bII). Thus, a single mechanism suffices for both three- and four-strand interactions. We will assume that the filament is orientated and grows by accretion at one end, which we will call the head end, and dissociates by loss from the tail end, the head to tail direction being set by the 3' (head) to 5' (tail) direction along the single strand. Once an initiation complex has established homologous contacts with a duplex, and the paired strands are surrounded by the spiral protein filament (Fig. 4aIII), the strand exchange reaction may proceed. We suggest it may occur at the terminal monomer at the tail end and depend upon allosteric changes associated with ATP hydrolysis, in which the bases are rotated out of their original Watson-Crick pairing to form heteroduplexes as shown in Fig. 4a(III) and (IV) and more clearly in a plane at right angles to the helical axis in Fig. 3a(III) and b(III). In space-filling molecular models, the bases are easily rotated into the required positions by rotation mainly about phosphate-oxygen bonds.

Since each RecA protein binds to more than one nucleotide, it may switch several base pairs at a time. Figure 4a(IV) and (V) show the terminal monomer switching 3 bp and then dissociating, leaving the next monomer to perform the same reac-

tion. Thus, we visualize that the spiral filament grows at the head end by monomers binding cooperatively, drawing the duplexes together, while monomers are released from the tail end as heteroduplex DNA is formed. The length of the spiral filament may therefore be variable, depending upon the balance of accretion at the head end dissociation from the tail end. The reaction will be completed when RecA protein, for whatever reason, fails to add further to the growing end, and the last monomer of the spiral filament dissociates.

Of the two heteroduplexes formed, the lower one should be free to coil into a relaxed duplex because of the single-strand gap, while the upper heteroduplex, having no gap, will be constrained but may be relaxed by topoisomerase I (Fig. 4a(V)). The reaction is completed by a switch in base stacking referred to as isomerization⁴ to provide the template needed for gap repair by DNA polymerase and ligase (Fig. 4a(V) and (VI)).

At this point, the gap has been repaired, the two duplexes are freed from the spiral filament, and they are joined by two single-strand cross-overs (Fig. 4a(VII)). They are converted to mature recombinants by the enzymatic resolution of cross-overs and the repair of mismatches in the heteroduplexes⁵⁰. The endonuclease activities performing these functions are still to be purified and studied.

Future prospects

Several features of the proposed enzymatic model require further investigation. The basic structure of homologous pairing has not been established. RecA binding to DNA and the structure of the resulting complex require further examination. Conflicting results on the stoichiometry of RecA binding need to be resolved. We do not know what triggers a strand exchange or how it takes place. We do not understand RecA binding or its release from DNA following strand exchange, nor why it uses so much ATP. The structure of RecA protein is still to be determined. Other enzymes, notably those for resolving single-strand cross-overs and for mismatch repair in heteroduplex DNA, are needed to fill important gaps in the enzymology of genetic recombination.

This work was supported by US PHS grants GM11014, CA26763, CA06519 and AM09397.

- Holliday, R. *Genet. Res.* **5**, 282-304 (1964).
- West, S. C., Cassuto, E. & Howard-Flanders, P. *Nature* **294**, 659-662 (1981).
- Radding, C. M. A. *Rev. Genet.* **16**, 405-437 (1983).
- Meselson, M. & Radding, C. M. *Proc. natn. Acad. Sci. U.S.A.* **72**, 358-361 (1975).
- Stahl, F. W. *Genetics Suppl.* **61**, 1-13 (1969).
- Stahl, F. W. *Genetic Recombination: Thinking about it in Phage and Fungi* (Freeman, San Francisco, 1979).
- Szostak, J. W., Orr-Weaver, T. L., Rothstein, R. J. & Stahl, F. W. *Cell* **33**, 25-35 (1983).
- Rupp, W. D., Wilde, C. E., Reno, D. L. & Howard-Flanders, P. *J. molec. Biol.* **61**, 25-44 (1971).
- Cassuto, E., West, S. C., Mursallim, J., Conlon, S. & Howard-Flanders, P. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3962-3966 (1980).
- Cunningham, R. P., DasGupta, C., Shibata, T. & Radding, C. M. *Cell* **20**, 223-235 (1980).
- West, S. C., Cassuto, E. & Howard-Flanders, P. *Molec. gen. Genet.* **187**, 209-217 (1982).
- Fogel, S., Mortimer, R., Lusnak, K. & Travers, F. *Cold Spring Harb. Symp. quant. Biol.* **43**, 1325-1341 (1979).
- Hamza, H., Haedens, V., Mekki-Berrada, A. & Rossignol, J. L. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7648-7651 (1981).
- Orr-Weaver, T. L., Szostak, J. W. & Rothstein, R. J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6354-6358 (1981).
- Strathern, J. N. *et al. Cell* **31**, 183-192 (1982).
- Lin, P. F., Bardwell, E. & Howard-Flanders, P. *Proc. natn. Acad. Sci. U.S.A.* **74**, 291-295 (1977).
- Clark, A. J. & Margulies, A. D. *Proc. natn. Acad. Sci. U.S.A.* **53**, 451-459 (1956).
- Gudas, L. J. & Pardee, A. J. *J. molec. Biol.* **101**, 459-477 (1976).
- McEntee, K., Hesse, J. E. & Epstein, W. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3979-3983 (1976).
- Gudas, L. J. & Mount, D. W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5280-5284 (1977).
- Emmerson, P. T. & West, S. C. *Molec. gen. Genet.* **155**, 77-85 (1977).
- Little, J. W. & Kleid, D. G. *J. biol. Chem.* **252**, 6251-6252 (1977).
- Roberts, J. W., Roberts, C. W. & Craig, N. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4714-4718 (1978).
- Horii, T., Ogawa, T. & Ogawa, H. *Proc. natn. Acad. Sci. U.S.A.* **77**, 313-317 (1980).
- Sancar, A., Stachelek, C., Konigsberg, W. & Rupp, W. D. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2611-2615 (1980).
- McEntee, K., Weinstock, G. M. & Lehman, I. R. *J. biol. Chem.* **256**, 8835-8844 (1981).
- West, S. C., Cassuto, E., Mursallim, J. & Howard-Flanders, P. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2569-2573 (1980).
- Stasiak, A., DiCapua, E. & Koller, T. *Cold Spring Harb. Symp. quant. Biol.* **47**, 811-820 (1983).

29. DiCapua, E., Engel, A. E., Stasiak, A. & Koller, T. *J. molec. Biol.* **157**, 87–103 (1982).
30. Stasiak, A. & DiCapua, E. *Nature* **299**, 185–186 (1982).
31. Dombrowski, D. F., Scraba, D. G., Bradley, R. B. & Morgan, A. R. *Nucleic Acids Res.* **11**, 7487–7504 (1983).
32. Chrysogelos, S., Register, J. C. & Griffith, J. J. *biol. Chem.* **258**, 12624–12631 (1983).
33. Flory, J. & Radding, C. M. *Cell* **28**, 747–756 (1982).
34. McKay, D. B., Steitz, T. A., Weber, I. T., West, S. C. & Howard-Flanders, P. *J. biol. Chem.* **225**, 6662 (1980).
35. Cox, M. M. & Lehman, I. R. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6018–6022 (1981).
36. Kahn, R., Cunningham, R. P., DasGupta, C. & Radding, C. M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4786–4790 (1981).
37. West, S. C., Cassuto, E. & Howard-Flanders, P. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6159–6153 (1981).
38. Roberts, J. W., Roberts, C. W., Craig, N. L. & Phizicky, E. M. *Cold Spring Harb. Symp. quant. Biol.* **43**, 917–923 (1979).
39. Ogawa, T. *et al. Cold Spring Harb. Symp. quant. Biol.* **43**, 909–915 (1979).
40. Shibata, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 1638–1642 (1979).
41. McEntee, K., Weinstock, G. M. & Lehman, I. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2615–2619 (1979).
42. Shibata, T. *et al. J. biol. Chem.* **256**, 7565–7572 (1981).
43. Shibata, T., Ohtani, T., Chang, P. K. & Ando, T. *J. biol. Chem.* **257**, 370–376 (1982).
44. Cox, M. M. & Lehman, I. R. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3433–3437 (1981).
45. DasGupta, C., Shibata, T., Cunningham, R. P. & Radding, C. M. *Cell* **22**, 437–446 (1980).
46. Cox, M. M. & Lehman, I. R. *J. biol. Chem.* **257**, 8523–8532 (1982).
47. Soltis, D. A. & Lehman, I. R. *J. biol. Chem.* **258**, 6073–6108 (1983).
48. West, S. C., Cassuto, E. & Howard-Flanders, P. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2100–2104 (1981).
49. DasGupta, C., Wu, A., Kahn, R., Cunningham, R. P. & Radding, C. M. *Cell* **25**, 507–516 (1981).
50. West, S. C., Countryman, J. K. & Howard-Flanders, P. *Cell* **32**, 817–829 (1983).
51. Gonda, D. K. & Radding, C. M. *Cell* **34**, 647–654 (1983).
52. Holloman, W. K. & Radding, C. M. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3910–3914 (1976).
53. West, S. C., Cassuto, E. & Howard-Flanders, P. *Nature* **290**, 29–33 (1981).
54. Wu, A. M., Bianchi, M., DasGupta, C. & Radding, C. M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1256–1260 (1983).
55. Iwabuchi, M., Shibata, T., Ohtani, T., Natori, M. & Seno, T. *J. biol. Chem.* **258**, 12394–12404 (1983).
56. McGavin, S. *J. molec. Biol.* **55**, 293–298 (1971).
57. McGavin, S. *Heredity* **39**, 15–25 (1977).
58. Wilson, J. H. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3641–3645 (1979).
59. Arnott, S., Bond, P. J., Selsing, E. & Smith, P. C. *J. Nucleic Acids Res.* **3**, 2459–2470 (1976).
60. DasGupta, C. & Radding, C. M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 762–766 (1982).
61. Bianchi, M. & Radding, C. M. *Cell* **35**, 511–520 (1983).
62. Livneh, Z. & Lehman, I. R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3171–3175 (1982).
63. Ohtani, T., Shibata, T., Iwabuchi, M., Nakagawa, K. & Ando, T. *J. Biochem. (Tokyo)* **91**, 1767–1775 (1982).
64. Shibata, T., Ohtani, T., Iwabuchi, M. & Ando, T. *J. biol. Chem.* **257**, 13981–13986 (1982).
65. Ohtani, T. *et al. Nature* **299**, 86–94 (1982).
66. Wu, A. M., Kahn, R., DasGupta, C. & Radding, C. M. *Cell* **30**, 37–44 (1982).

ARTICLES

Sedimentation under deep-sea storms

C. D. Hollister* & I. N. McCave**

* Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

† School of Environmental Sciences, University of East Anglia, Norwich NR47TJ, UK

Fast currents, high concentrations of suspended sediment and grooved mud beds are associated with erosion in frequent abyssal storms where a fast deep mean flow is augmented (or reversed) by intense intermittent currents. This occurs about 5 km below the Gulf Stream or its rings. The waning phase of a storm results in development of bedforms and rapid deposition of a mud blanket. Several other regions of the world ocean display evidence of abyssal storm activity.

REPEATED sampling of an area at 4.8–4.9 km depth lying under the path of the Gulf Stream or its warm-core rings revealed extremely high current velocities (up to 0.73 m s^{-1} (ref. 1)), suspended sediment concentrations up to 12 g m^{-3} (ref. 2) and bedform development. All these varied in time. The area is the lower continental rise south of Halifax, Nova Scotia (Fig. 1), and the measurements were taken as part of the High Energy Benthic Boundary Layer Experiment (HEBBLE)^{3–5}. The experiment aimed to develop and test a coupled bottom Ekman layer and sediment transport model for cohesive sediment over uniformly distributed bed roughness⁵. Regional investigations in HEBBLE have provided numerous hydrographic^{6,7}, photographic⁸, sedimentological⁹, current velocity^{1,10–12}, light transmission^{13–15}, and suspended sediment^{2,16,17} data. These have been used to select a $4 \times 2 \text{ km}$ box on the lower continental rise at $40^{\circ}27' \text{ N}$ $62^{\circ}20' \text{ W}$ where bed roughness is uniform, bed slopes are low ($<1:100$) and currents are intermittently fast (Fig. 1). The Nova Scotian Rise was first investigated because earlier data on sediments, photographs and currents suggested active sediment transport in the area^{18–20}.

Most sediment is carried to the depth of the continental rise by debris flow and turbidity currents. Subsequently, bottom currents produce two well-defined deposit types: those dominated by reworked current-deposited sediment, exemplified by parts of the eastern margin of the United States^{21,22}, and those where current reworking is slight, such as the margin off north-west Africa^{23–26}. The lower Scotian Rise lies downstream of the Laurentian Fan, a major sediment accumulation produced by downslope sediment transport^{27,28}. This is a probable source of fine material moving over the HEBBLE site in a deep western boundary current (which is not the 'western boundary undercurrent' comprising Norwegian Sea overflow water; this occurs above 4,000 m on the rise^{10,29}, Fig. 1a).

Here we draw together diverse data bearing on the rapid erosion, transport and deposition of sediments on the lower Nova Scotian Rise and on the driving forces, a combination of a strong mean component (DC current) with strong fluctuating components (AC current). We then examine the implications for sediment dispersal and deposition in the deep sea.

Sedimentology and stratigraphy

The $2 \times 4 \text{ km}$ HEBBLE box (Fig. 1b) was sampled with more than 30 box cores in July 1982. Over this area the upper unit is a $\sim 4\text{-cm}$ layer of soft brown mud with a silt/clay ratio of 1.70, foraminiferal sand content of 6% and water content (% wet weight) of $\sim 50.0\%$. The thickness of this layer measured in cores and on X-radiographs varies between $\sim 0.5 \text{ cm}$ and 12.5 cm . Its vane shear strength is uniformly low at $\sim 0.4 \text{ kPa}$, rising rapidly to $\sim 4 \text{ kPa}$ in the underlying layer of foraminiferal silt³⁰, whose water content decreases from 45.8% at the top of the layer to 36.9% at a depth of 14 cm. This underlying unit is about 30 cm thick, is extensively burrowed (live worms have been found down to the base of the unit) and is probably all Holocene in age as it contains *Globorotalia menardii*. The foraminiferal mud contains 1–30% sand, chiefly of planktonic foraminifera. Two longer cores have a gravelly mud of presumed late Pleistocene age beneath the Holocene.

Internally, the surface mud unit shown in Fig. 2 is variably burrowed and laminated. Some burrows are large and terminate in mounds, others are a filamentous network. The uniform physical properties of this surface layer suggest rapid deposition. This is corroborated by ²³⁴Th ($t_{1/2} = 24 \text{ days}$) distributions showing penetration of the radionuclide to depths as great as 6 cm (ref. 31). A balance must be struck between introduction of this material by advection (sediment deposition) and (biological) mixing, parameterized as a diffusivity^{32,33}. The best estimate

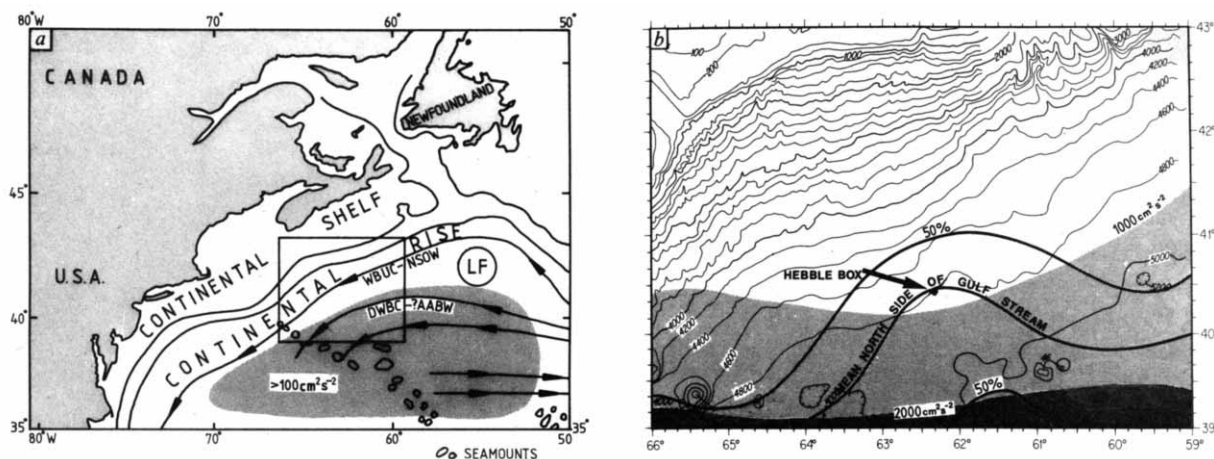


Fig. 1 Location of the HEBBLE box on the Nova Scotian continental rise. *a*, Physiography of the region with the paths of the Western Boundary Undercurrent (WBUC) transporting Norwegian Sea Overflow Water (NSOW) and a Deeper Western Boundary Current (DWBC) transporting Antarctic Bottom Water (AABW) origin^{6,29}, and, stippled, the region of high abyssal kinetic energy ($>100 \text{ cm}^2 \text{ s}^{-2}$)^{38,39}. *b*, Contoured enlargement of the slope-rise province with location of the HEBBLE box, the mean position of the northern side of the Gulf Stream^{7,3} and its $\pm 50\%$ limits, and, shaded, the region of high surface kinetic energy ($>1,000 \text{ cm}^2 \text{ s}^{-2}$)⁷⁴. Bathymetry of *b* courtesy of Dr A. N. Shor. LF, Laurentian Fan.

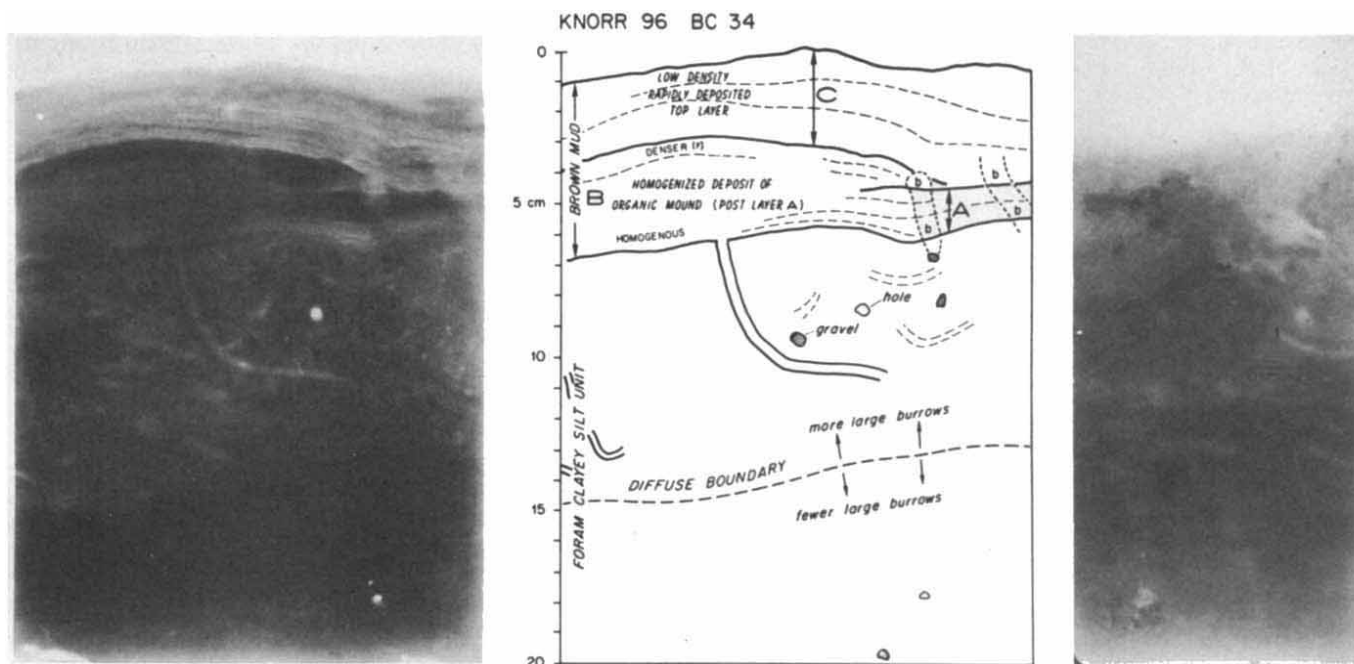


Fig. 2 Left: X-radiograph of the uppermost sediments in Knorr Cruise 96 (July 1982) box core 34. Centre: interpretation of the internal structure showing two depositional events (A and C) separated by an episode of mound construction (B). Right: an X-radiograph from box core 2 showing burrow-mixed upper part of the sediment column containing fine gravel (dark grains).

from one site is a deposition rate of $1.42 \text{ cm per month}$ (17 cm yr^{-1}) and mixing parameter of $85 \text{ cm}^2 \text{ yr}^{-1}$ (ref. 30), values confirmed and extended by analysis of more samples by our colleagues D. DeMaster and C. Nittrouer³¹. These extremely high rates require very high suspended sediment concentrations ($\sim 100 \text{ g m}^{-3}$) occurring intermittently over a few months³⁰. Deposition is often followed by rapid biological mixing of the sediment, a feature well shown by some X-radiographs (Fig. 2).

Detailed size analysis of 20 samples from 1–5 mm depths taken in July 1982 and June 1983 reveal a marked change in the material present. At depth, below the surface brown mud, on both occasions the silt fraction ($2\text{--}63 \mu\text{m}$) contained two modes at $\sim 4 \mu\text{m}$ and $\sim 14 \mu\text{m}$. However, the surface 4 cm in July 1982 showed only a single peak at $\sim 14 \mu\text{m}$ (ratio of % at $14 \mu\text{m}$ to % at $4 \mu\text{m} = 2.99 \pm 1.38$) whereas in June 1983 the

bed was quite different with a clearly bimodal size distribution and a peak-height ratio of 1.42 ± 0.24 ($\pm 1 \text{ s.d.}$). The unimodal surface layer deposited before July 1982 must have been laid down in conditions of higher shear stress, suppressing deposition of the finer mode. Taking the preliminary *in situ* settling velocities (from trials of the optical tube of R. Zaneveld) for the fine mode to be $5.5 \times 10^{-4} \text{ cm s}^{-1}$, the boundary shear stress under which the 1982 bed was deposited, preventing deposition of $4 \mu\text{m}$ silt, was $>0.005 \text{ Pa}$ (shear velocity $>0.22 \text{ cm s}^{-1}$), while the 1983 bed was laid down under a lower stress³⁴. Many X-radiographs reveal a laminated upper 5 cm of the bed, thus the bimodal deposit of 1983 could not have been formed by biological mixing-in of fine sediment deposited on top of the 1982 layer. We conclude that the bed of July 1982 had been completely eroded and that a new deposit of 'bimodal mud' up to 4 cm thick had formed since then. Again, our inference is

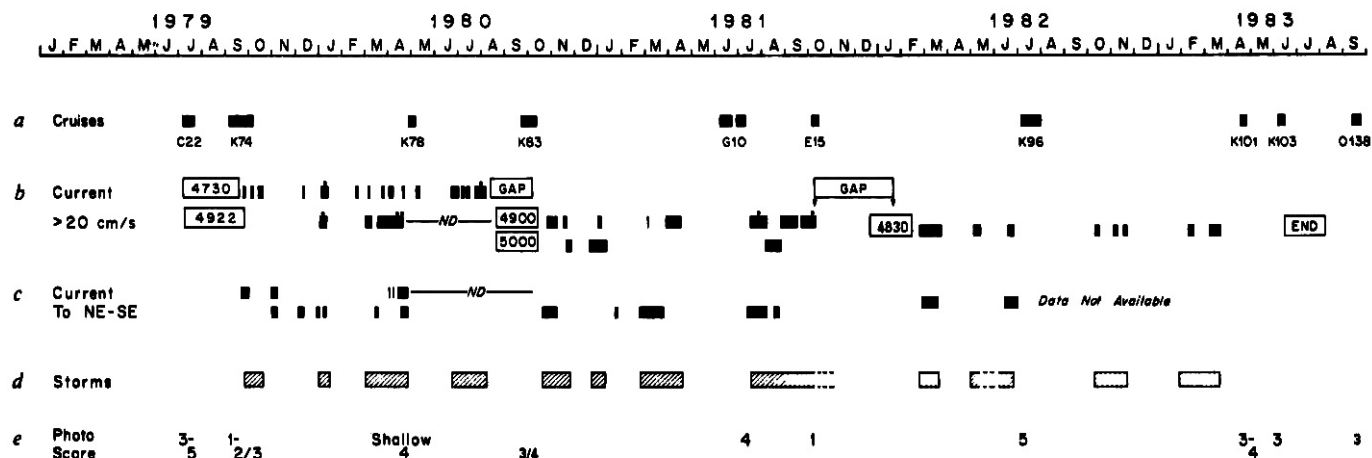


Fig. 3 A time-series of: *a*, cruises to the Nova Scotian rise in HEBBLE by Research Vessels C = *Conrad*, K = *Knorr*, G = *Gyre*, E = *Endeavour*, O = *Oceanus*; *b*, times when the currents were $>0.20 \text{ m s}^{-1}$ and, with a dot above the block, $>0.40 \text{ m s}^{-1}$ (courtesy of Dr M. Wimbush and Dr G. Weatherley; data for October 1980 to June 1982 in Weatherley and Kelley⁶ and Koenig *et al.*⁸); *c*, times when currents flow to the east indicating eddy reversal of the mean flow, from the data sources for speed; *d*, times when the combination of speed and direction can be called a storm—this includes some lulls in the storm(s); *e*, scores (based on Table 1 criteria) of the suites of photographs from the vicinity of the HEBBLE box for cruise G10 onwards, for the deepest stations of K78, and for a 4,950 m station for K83. The range of C22 and K74 scores is discussed in the text.

one of extremely rapid erosion and deposition in this area, at rates which would not be thought low in an estuarine environment.

Photographs and currents

We have had at least one cruise per year to the Nova Scotian Rise since 1979, returning photographs from between 4,750 and 4,950 m (Fig. 3). Gross bedform zonation of the rise shows evidence of more tranquil conditions where the bed is shallower than 4,500 m and of faster currents below 4,750 m with very common longitudinal ripples below 4,850 m^{8,35}. The zone between bottom depths of 4,750 and 4,950 and from 61 to 64°W was photographed in July and September 1979. These and the subsequent photographs can be assigned to a position in a rough scale of relative importance of erosion or deposition on the basis of the many features observed (Table 1) (compare with ref. 36, p. 357). It is not possible to calibrate this scale properly, though grooving (a score of 1) observed in October 1981 was associated with currents which exceeded 0.4 m s^{-1} in the preceding 2 weeks (Fig. 3), and the tranquil end (a score of 5) has currents less than $\sim 0.05 \text{ m s}^{-1}$. It is unlikely that velocities for scores 2 to 4 can be obtained by linear interpolation.

The large-scale spatial variability displayed by the 1979 photographs also contains a strong temporal signal. In July the area was all at the depositional end of the scale, from 3 to 5 on the criteria of Table 1, with the scores of 4 and 5 occurring at shallower depths and 3 at depths over 4,900 m. In September the photographs were taken during a period of strong currents. Two of the stations between 61 and 61°30' W at 4,840 and 4,920 m showed pronounced grooving, exhumed tubes and other indicators of erosion. Other stations rating scores of 1 to 2 occur along the south (deeper) side of the photographed region. The lowest scores at this time are 2 to 3 at 4,830 m depth. The most intense effects were seen in deeper water with intense scouring at two stations in the east of the region. A circular area incorporating the two scoured stations would be $\sim 10^3 \text{ km}^2$ but (the affected region might be more extensive along isobaths).

From 1981 onwards all photographs were precisely navigated by bottom transponder in the HEBBLE box. They display enormous temporal variability (Figs 3, 4), ranging from the most severe erosion to tranquil deposition with organic reworking, ratings of 1 to 5 on the criteria of Table 1. The end members are represented by the areas photographed in October 1981 on *Endeavour* 75 (score 1) and July 1982 on *Knorr* 96 (score 5)

(Fig. 4a, c). Over an area the size of the HEBBLE box the photographed bed condition is spatially very uniform at a given time. During *Knorr* 96 the 16 sub-areas had over 20 photographs taken in each, and none rated a score less than 4. Current velocities measured about 35 km south of the EN 75 photographs during the previous 10 days (1–8 October) were all $>0.30 \text{ m s}^{-1}$ on daily average, and for 2 days they were $>0.40 \text{ m s}^{-1}$ (ref. 6). During *Knorr* 96 the photographs show surfaces with mounds, scattered faecal pellets, organic tracks and trails, various animal holes in the mud and occasional fine lineations. For the week before the start of the photographic survey, currents at 59 m above bottom (mab) in the HEBBLE box were $<0.10 \text{ m s}^{-1}$ and for half the time they were $<0.06 \text{ m s}^{-1}$.

At intermediate speeds, 0.05 to 0.15 m s^{-1} , deposition of silt occurred with development of barchan-like ripples in $\sim 15 \mu\text{m}$ silt containing $\sim 25\%$ clay and $<10\%$ sand (Fig. 4b). The steeper faces on these bedforms suggest a measure of non-cohesive behaviour on the part of these clayey silts. The barchan ripples grow on the downstream side of biogenic mounds. It is not clear what dynamical condition controls the difference between formation of either single tails (mound-and-tail structure) or 'swallow-tailed' barchan ripples downstream of mounds. These

Table 1 Photographic features indicative of erosion/deposition

Strong erosion	2	3	4	Tranquil deposition
1				5
Scraped grooves				
Exhumed tubes				
Winnowed sand sheet				
Crag and tail				
	Barchan ripples			
	Cornices/slip faces			
	Lineations			
		Smoothed surface		
		Mounds and tails		
		5-Holes and fairy rings		
		Animal tracks		
		Ampharetid pits		
		Symmetric mounds		
		Clear water		
			Organic surface debris	

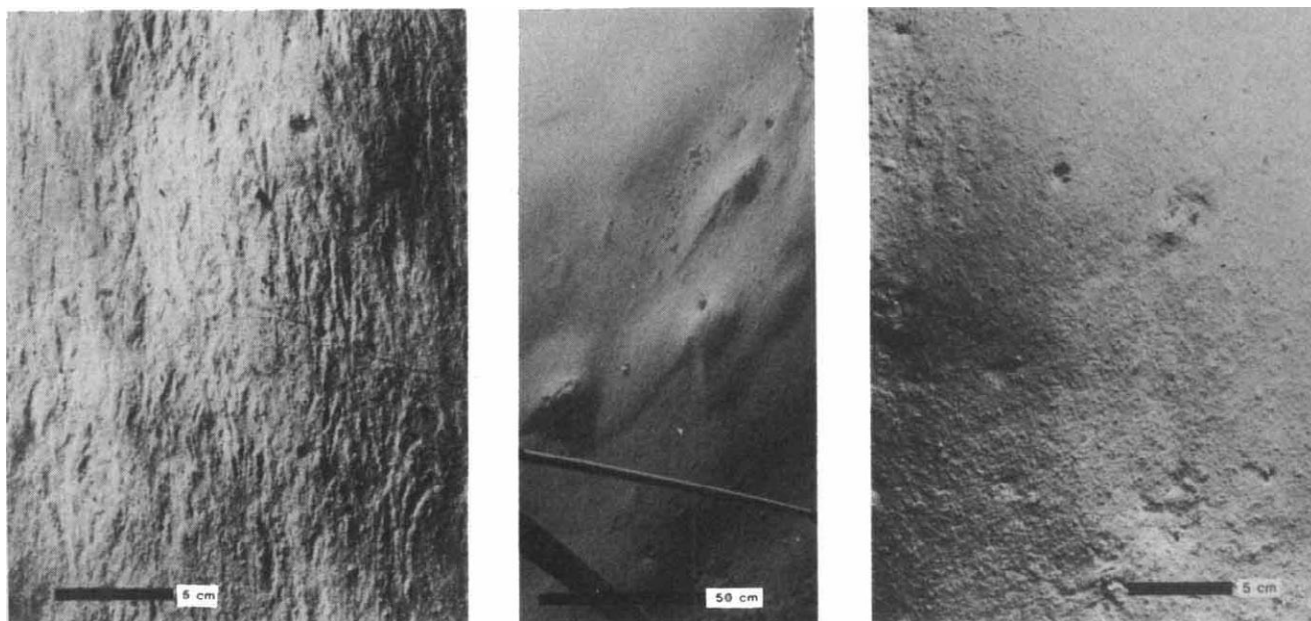


Fig. 4 Representative photographs for the conditions of: left, major erosion yielding a grooved bed (EN75); centre, deposition under a moderate current yielding barchan ripples in clayey silt (KN103); and right, tranquil conditions with a biologically textured bed and little evidence of current activity (KN96). All from the area of the HEBBLE box.

conditions are exemplified by the photographs of April and June 1983.

On the basis of the stronger bottom currents and direction reversals shown on Fig. 3 we have picked off certain times that we call 'storms'. We define as abyssal storms those periods of several days to a few weeks with intermittently high current speeds (over 0.20 m s^{-1} with peaks over 0.30 m s^{-1}) and often with reversals of current direction (see current meter records in Weatherly and Kelley¹⁰). Associated with this perturbation of the velocity field is high turbidity caused by resuspension of bottom sediments which some authors have termed storms^{15,37}. We prefer to view the increased turbidity as a common consequence of storminess. There are about three of these storms per year occupying about 35% of the time. Less than a third of them contain speeds over 0.40 cm s^{-1} at the HEBBLE site, though they may contain such speeds somewhere in the vicinity. Nevertheless, an area can expect, on average, to suffer one of the intense scouring events like that photographed in October 1981, once per year, and be subject to a scale 2 or 3 event twice per year. The material put into suspension is deposited (or dispersed) in the remaining 65% of the time. The deposition is probably as episodic as the erosion, since concentration controls deposition rate both directly and through its control of settling velocity, thereby ensuring that initial deposition rates after storms will be fast.

The rates of deposition suggest that the upper layer 'C' of the sediment column shown in Fig. 2, 2.5–4 cm in thickness, was probably deposited between the minor storm of March 1982 and the sampling date of July 1982. The first layer 'A' probably post-dates the intense October 1981 storm, putting the mound formation in the period December to February 1982. These rates of up to a centimetre per month deposition are consistent with the ^{234}Th dating mentioned above^{30,31}. The two-event signature of box core 34 (Fig. 2) is shown in at least 5 of the other 15 sub-areas of the HEBBLE box. The stratigraphy of the uppermost sediment column here is extremely ephemeral and its chance of being preserved very small. The overall Holocene deposition rate on this part of the continental rise is $\sim 5.5 \text{ cm kyr}^{-1}$ (giving an annual deposition/preservation ratio of 3,100), and the deposits preserved are almost totally disturbed by burrowing animals.

Driving mechanisms

The area we describe has three abyssal features possessed by several other points in the world ocean: (1) strong mean flow of $0.10\text{--}0.15 \text{ m s}^{-1}$ (refs 10, 29); (2) high variability of flow speed and direction resulting in a high eddy kinetic energy (variance of speed)^{38–40}; and (3) a large, fresh supply of mud in the form of turbidity current deposits located upstream (the Laurentian Fan). Several other sites with large supplies of mud and strong steady currents have been examined elsewhere, for example, Feni, Hatton and Gardar drifts in the northeastern Atlantic^{41,42}, and Blake and Greater Antilles Outer Ridge in the southwestern North Atlantic^{43,44}. In none of these locations have the extraordinarily high turbidities ($>1 \text{ g m}^{-3}$) and flow speeds attained on the Scotian Rise been matched.

The high turbidities attained must be related to high rates of erosion, as Armi⁴⁵ shows that they are not due to turbidity currents, an earlier explanation⁴⁶. An important determinant of this is the sediment water content^{47,48}. Rapidly deposited sediments have high water contents, and in the HEBBLE area the water content of the surface 2 cm ranges from $>100\%$ dry weight ($>50\%$ wet weight) to 170% (61% wet weight) (J. Y. Yingst and A. J. Silva, personal communication). This high potential erodability is a consequence of the fluctuating velocity regime, which allows rapid deposition of material under the low velocities following a storm. By contrast an area which suffers a more sustained stress may become 'stress-hardened' and more difficult to erode, requiring a large fluctuation in speed to move it^{49,50}. Of course, the fact that the maximum currents here are up to 0.73 m s^{-1} , almost twice as high as those flowing over other deep-sea sedimentary bodies, also helps cause rapid erosion.

The origin of the high abyssal eddy kinetic energy in this region appears to be related to the high surface eddy kinetic energy of the Gulf Stream overhead^{38–40,51,52}. However, Weatherly³⁹ shows that proximity of the Gulf Stream or its warm-core rings to the deep current meter locations is associated with a high frequency of current reversals (easterly flows) and with mean speeds of 0.15 to 0.20 m s^{-1} to both west and east. Rhines⁵³ numerical models suggest a uniform decrease of the ratio of surface to bottom velocity with increasing E/δ where

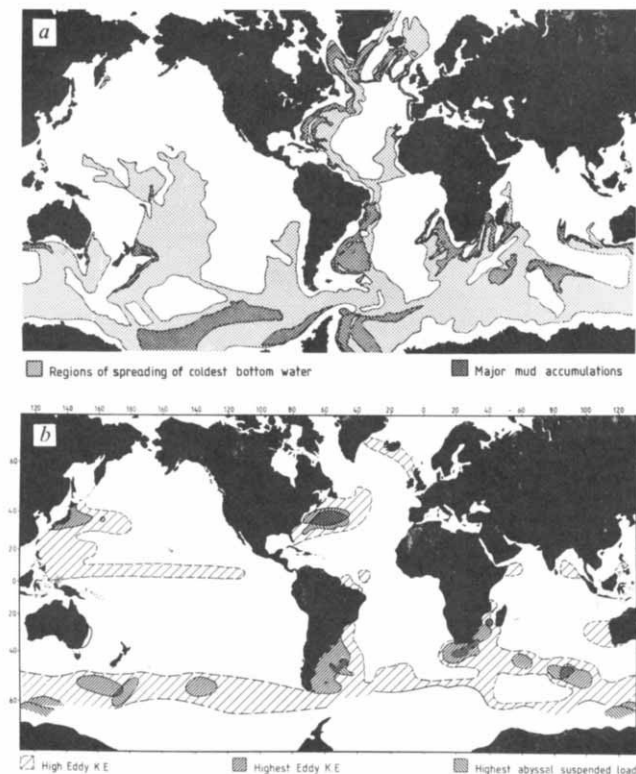


Fig. 5 *a*, Regions of spreading of cold bottom water⁶⁷ (light stipple) superimposed on distribution of large current-controlled accumulations of mud⁶⁸ (heavy stipple). *b*, Areas of high surface eddy kinetic energy or variability of surface elevation, based on Wyrki *et al.*⁷¹ and Cheney *et al.*⁶⁹, with, superimposed, the region of highest suspended particulate load ($>20 \text{ g m}^{-2}$) in the North and South Atlantic from Biscaye and Eitrem⁶⁴, the maximum turbidity in the Indian Ocean from Kolla *et al.*⁶⁵ and the turbidity high south of New Zealand from Dr P. E. Biscaye.

E is the Rossby number and δ is the relative roughness, a feature demonstrated by Dickson's results⁴⁰. At high energy levels the eddies should be more barotropic⁴⁰ and the flow should depend only weakly on depth as shown in the Gulf Stream area by Schmitz⁵⁴. Looking at the western Atlantic as a whole, Schmitz³⁸ identifies regions of deep strong mean flow as ones of enhanced eddy kinetic energy though the values are only up to half those found under the Gulf Stream.

Implications

(1) The deep-sea stratigraphical record under sites of high eddy kinetic energy is much more fragmentary than generally supposed. The situation more closely resembles storm-dominated shelf sedimentation than the continuous accumulation presumed for many parts of the deep ocean. In the Scotian Rise area the mean deposit thickness of 10^3 yr net accumulation can be removed in a few weeks and redeposited in a few months. The high nutrient flux resulting from both horizontal advection and vertical flux from the Gulf Stream's surface productivity allows intensive activity by burrowing organisms⁹, yielding phenomenally high values of biological diffusivity ($3\text{--}85 \text{ cm}^2 \text{ yr}^{-1}$)^{30,31} compared with previous deep-sea values (all $<0.5 \text{ cm}^2 \text{ yr}^{-1}$). Thus the signal of intermittent erosion and deposition is largely smeared out or erased once the sediments have passed below the zone of episodic erosion.

(2) This region is clearly important for generation of nepheloid layers and for their thickening by detachment and injection of turbid bottom-mixed layers to points higher up in the water column^{13,16}. The high horizontal eddy kinetic energy may also be accompanied by enhanced vertical eddy kinetic energy and thus higher vertical turbulent diffusivity. It has been suggested that the cross-isopycnal diffusivity of $1\text{--}3 \text{ cm}^2 \text{ s}^{-1}$ identified by

Hogg *et al.*⁵⁵ and Whitehead and Worthington⁵⁶ also results in the thickening of the nepheloid layer above the bottom-mixed layer through upwards diffusion of small particles^{16,57}. A major feature of the GEOSECS suspended sediment profile down the western Atlantic is relatively high concentration extending up to a depth of 2,000 m ($>2,000 \text{ m.a.b.}$) between 35° N and 45° N ⁵⁸. This may contain material resuspended from the bed under high eddy kinetic energy of the Gulf Stream and warm-core rings on the continental rise or cold-core rings over northern Bermuda Rise, which has been diffused upwards, as well as sediment that has spread laterally along isopycnals. Below the surface layer the small particles (mainly $<5 \mu\text{m}$) providing most of the light-scattering signal to deep-sea nephelometers have a long residence time in suspension, probably more than a year^{16,59}. Significant amounts of the optically detected material in nepheloid layers may thus have an origin in resuspension far from the detection point. This point is supported by the chemical data of Bishop and Biscaye⁶⁰, who find similarity between suspended and bed material in areas of strong currents where resuspension is likely, but some dissimilarity in quiescent regions with weak nepheloid layers. There the bottom sediment results mainly from rapid vertical flux from the surface and the nepheloid layer contributes only a slight rate of deposition. The stormy areas of the sea bed are seen as major sources of suspended material which is raised up and then advected across ocean basins. This situation is roughly analogous to that of soil aerosols, a major portion of which originate from a few areas with erodible soils and intermittently strong winds^{61,62}.

(3) The points of major resuspension under abyssal storms in the world ocean are likely to be those where surface eddy kinetic energy is high and coupled to a strong near-bottom mean flow and sufficient mud. An extremely dense nepheloid layer would indicate the possibility of storm activity and large sediment drifts and mud waves indicate significant long-term deposition. Several authors using the Lamont nephelometer have demonstrated a clear relationship between the regions of high mean velocity in deep boundary currents and high suspended sediment content^{63–66}. Regions of spreading of coldest bottom water⁶⁷ and areas of depositional sedimentary bedforms⁶⁸ correspond closely (Fig. 5*a*). The distribution of eddy kinetic energy inferred from satellite^{69,70} and ship drift observation⁷¹ shows several areas of elevated surface eddy kinetic energy and a few particularly high spots where we presume high abyssal eddy kinetic energy. These are the North American basin under the Gulf Stream system, the Argentine basin under the confluence of the Falkland and Brazil currents, under the Kuroshio off Japan, in areas off South Africa in the Natal Valley and Mozambique Basin beneath the Agulhas current, in the Tasman Sea under the East Australian current, and over large areas beneath the Antarctic Circumpolar Current—south of the Crozet Plateau, east of the Kerguelen Plateau, in the Weddell Sea and on the southwestern side of the New Zealand Plateau (Fig. 5*b*). Many of the areas of potential bottom storminess are also the regions of maximum turbidity in the ocean basins (Fig. 5*b*). In the Atlantic the two maxima are under the Gulf Stream and in the Argentine Basin^{63,64}. In the Indian Ocean maxima coincide with the Agulhas region and the area east of Kerguelen⁶⁵. South of New Zealand along the southern end of Chatham Rise, P. Biscaye (personal communication) has located the maximum turbidity in the Pacific. The notable exception to this list is the area under the Kuroshio where no great source of suspended material has been detected⁷². The large amount of sediment eroded from Japan goes into the Japan trench and there is no strong near-bottom thermohaline flow in the region. The Pacific equatorial high surface kinetic energy does have a substantial accumulation of biogenic mud beneath it, but neither there nor in the Indian Ocean are there strong thermohaline currents along the bottom. Although only two areas under the Antarctic circumpolar current have demonstrated extremes of turbidity there is a general paucity of data from the region. It may be among the most stormy (at the bottom as well as the top) in the ocean and act as a major source of suspended material to

the abyssal thermohaline flows emanating from there and penetrating the western sides of the ocean basins.

These results drive us towards a fundamental revision of the notion that "... the warm water sphere protects and isolates the abyss from the more familiar forces of weather, leaving as the only significant dynamic factors in the nearly closed abyssal system the thermohaline currents...' (ref. 36, p. 371).

We thank our colleagues in HEBBLE for their efforts, particularly Drs G. L. Weatherly and M. Wimbush for current

meter data, Dr J. Yingst for X-radiography, Professor A. J. Silva for geotechnical data and the NCSU group for radiochemistry. Discussions with Dr W. J. Schmitz on abyssal eddy kinetic energy were very helpful, and we thank him and Weatherly for preprints of their work on abyssal kinetic energy under the Gulf Stream. This work was supported by the US Office of Naval Research under contract N00014-82C-0019 NR-083 to Woods Hole Oceanographic Institution. Woods Hole contribution 5570.

Received 24 October 1983; accepted 17 January 1984.

1. Richardson, M. J., Wimbush, M. & Mayer, L. A. *Science* **213**, 887-888 (1981).
2. Biscaye, P. E. *et al. EOS* **61**, 1014 (1980).
3. McCave, I. N., Hollister, C. D. & Pyle, T. E. *Woods Hole Oceanogr. Inst. tech. rep. WHOI-78-48* (1978).
4. Hollister, C. D., Nowell, A. R. M. & Smith, J. D. *Woods Hole Oceanogr. Inst. tech. rep. WHOI-80-32* (1980).
5. Nowell, A. R. M., Hollister, C. D. & Jumars, P. A. *EOS* **63**, 593-595 (1982).
6. Weatherly, G. L. & Kelley, E. A. *Florida St. Univ. Dep. Oceanogr. Tech. Rep.* (1981).
7. Weatherly, G. L., Kelley, E. A. & Harkema, R. *Florida St. Univ. Dep. Oceanogr. tech. Rep.* (1981).
8. Tucholke, B. E. *Nature* **296**, 735-737 (1982).
9. Yingst, J. Y. & Aller, R. C. *Mar. Geol.* **48**, M7-M15 (1982).
10. Weatherly, G. L. & Kelley, E. A. *J. mar. Res.* **40**, 985-1012 (1982).
11. Kelley, E. A., Weatherly, G. L. & Evans, J. C. *J. phys. Oceanogr.* **12**, 1150-1153 (1982).
12. Koeng, P., Harkema, R. & Weatherly, G. L. *Florida St. Univ. Dep. Oceanogr. tech. Rep.* 83-01 (1983).
13. Spinrad, R. & Zaneveld, J. R. V. *J. geophys. Res.* **87**, 9553-9561 (1982).
14. Pak, H. *Mar. Geol.* **51**, 77-97 (1983).
15. Pak, H. & Zaneveld, J. R. V. *J. geophys. Res.* **88**, 4427-4432 (1983).
16. McCave, I. N. *J. geophys. Res.* **88**, 7647-7666 (1983).
17. Spinrad, R., Zaneveld, J. R. V. & Kitchen, J. C. *J. geophys. Res.* **88**, 7641-7645 (1983).
18. Hollister, C. D. thesis, Columbia Univ. (1967).
19. Zimmerman, H. B. *J. geophys. Res.* **76**, 5865-5876 (1971).
20. Hollister, C. D. & Heezen, B. C. in *Studies in Physical Oceanography* Vol. 2 (ed. Gordon, A. L.) 37-66 (Gordon & Breach, New York, 1972).
21. Heezen, B. C., Hollister, C. D. & Ruddiman, W. F. *Science* **152**, 502-508 (1966).
22. Schneider, E. D., Fox, P. J., Hollister, C. D., Needham, D. & Heezen, B. C. *Earth planet. Sci. Lett.* **2**, 351-359 (1967).
23. Embley, R. *Geology* **4**, 371-374 (1976).
24. Embley, R. & Jacobi, R. *Mar. Geotech.* **2**, 205-228 (1977).
25. Jacobi, R. & Hayes, D. E. in *Geology of the Northwest African Continental Margin* (eds von Rad, U., Hinz, K., Sarnthein, M. & Seibold, E.) 182-212 (Springer, Heidelberg, 1982).
26. von Rad, U. & Wissman, G. in *Geology of the Northwest African Continental Margin* (eds von Rad, U., Hinz, K., Sarnthein, M. & Seibold, E.) 106-131 (Springer, Heidelberg, 1982).
27. Stow, D. A. V. *Bull. Am. Ass. Petrol. Geol.* **65**, 375-383 (1981).
28. Uchupi, E. & Austin, J. A. *Can. J. Earth Sci.* **16**, 1726-1752 (1979).
29. Hogg, N. G. *Deep-Sea Res.* **30**, 945-961 (1983).
30. McCave, I. N. *et al. Mar. Geol.* (in the press).
31. DeMaster, D. J., McKee, B. A. & Nittrouer, C. A. *EOS* **63**, 991 (1982).
32. DeMaster, D. J. & Cochran, J. K. *Earth planet. Sci. Lett.* **61**, 257-271 (1982).
33. Goldberg, E. D. & Koide, M. *Geochim. cosmochim. Acta* **26**, 417-450 (1962).
34. McCave, I. N. & Swift, S. A. *Bull. geol. Soc. Am.* **87**, 541-546 (1976).
35. Tucholke, B. E. & Hollister, C. D. *Mar. Geol.* (in the press).
36. Heezen, B. C. & Hollister, C. D. *The Face of the Deep* (Oxford University Press, 1971).
37. Gardner, W. D. & Sullivan, L. G. *Science* **213**, 329-331 (1981).
38. Schmitz, W. *J. mar. Res.* (submitted).
39. Weatherly, G. L. *J. mar. Res.* (submitted).
40. Dickson, R. R. in *Eddies in Marine Science* (ed. Robinson, A. R.) 278-353 (Springer, Heidelberg, 1983).
41. Jones, E. J. W., Ewing, M., Ewing, J. I. & Eittrheim, S. L. *J. geophys. Res.* **75**, 1655-1680 (1970).
42. McCave, I. N., Lonsdale, P. F., Hollister, C. D. & Gardner, W. D. *J. sedim. Petrol.* **50**, 1049-1062 (1980).
43. Biscaye, P. E. & Eittrheim, S. L. in *Suspended Solids in Water* (ed. Gibbs, R.) 227-260 (Plenum, New York, 1974).
44. Tucholke, B. E. *J. Geol.* **83**, 177-207 (1975).
45. Armi, L. *Science* **208**, 1061-1062 (1980).
46. Amos, A. F. & Gerard, R. D. *Science* **203**, 894-897 (1979).
47. Migniot, C. *La Houille Blanche* **7**, 591-620 (1968).
48. Thorn, M. F. C. & Parsons, J. G. in *Proc. 3rd Int. Symp. Dredging Technol.*, 349-358 (Br. Hydraul. Res. Assoc., Cranfield, 1980).
49. McCave, I. N. in *Geol. Soc. Lond. Spec. Publ.* (in the press).
50. Mitchell, J. K. *Fundamentals of Soil Behaviour*, 291 (Wiley, New York, 1976).
51. Schmitz, W. J., Robinson, A. R. & Fuglister, F. C. *Science* **170**, 1192-1194 (1970).
52. Richardson, P. L. *J. geophys. Res.* **88**, 2705-2709 (1983).
53. Rhines, P. B. in *The Sea* Vol. 6 (eds Goldberg, E. D. *et al.*) 189-318 (Wiley-Interscience, New York, 1977).
54. Schmitz, W. J. *J. mar. Res.* **38**, 111-133 (1980).
55. Hogg, N. G., Biscaye, P. E., Gardner, W. D. & Schmitz, W. J. *J. mar. Res.* **40** (Suppl.), 231-263 (1982).
56. Whitehead, J. A. & Worthington, L. V. *J. geophys. Res.* **87**, 7903-7924 (1982).
57. Eittrheim, S. L. & Ewing, M. in *Studies in Physical Oceanography* Vol. 2 (ed. Gordon, A. L.) 123-167 (Gordon & Breach, New York, 1972).
58. Brewer, P. G. *et al. Earth planet. Sci. Lett.* **32**, 393-402 (1976).
59. McCave, I. N. *Deep-Sea Res.* **31** (in the press).
60. Bishop, J. & Biscaye, P. E. *Earth planet. Sci. Lett.* **58**, 265-275 (1982).
61. Jackson, M. L. *et al. Soil Sci.* **116**, 135-145 (1973).
62. Gillette, D. A., Patterson, E. M., Prospero, J. M. & Jackson, M. L. in *Aerosols and Climate* (ed. Robinson, E. D.) (US natn. Oceanic & Atmospheric Admin., submitted).
63. Eittrheim, S., Thorndike, E. M. & Sullivan, L. *Deep-Sea Res.* **23**, 1115-1127 (1976).
64. Biscaye, P. E. & Eittrheim, S. L. *Mar. Geol.* **23**, 155-172 (1977).
65. Kolla, V., Sullivan, L. G., Streeter, S. S. & Langseth, M. *Mar. Geol.* **21**, 171-189 (1976).
66. Kolla, V., Henderson, L., Sullivan, L. & Biscaye, P. E. *Mar. Geol.* **27**, 1-17 (1978).
67. Mantyla, A. W. & Reid, J. L. *Deep-Sea Res.* **30**, 805-833 (1983).
68. Heezen, B. C. & Sharp, M. in *World Ocean Floor* (Tharp, M.) (Lamont-Doherty Geological Observatory, 1977).
69. Cheney, R. E., Marsh, J. G. & Beckley, B. D. *J. geophys. Res.* **88**, 4343-4354 (1983).
70. Douglas, B. C., Cheney, R. E. & Agreen, R. W. *J. geophys. Res.* **88**, 9595-9603 (1983).
71. Wyrki, K., Magaard, L. & Hager, J. *J. geophys. Res.* **81**, 2641-2646 (1976).
72. Ewing, M. & Connary, S. D. *Geol. Soc. Am. Mem.* **126**, 41-82 (1970).
73. Fisher, A. *Gulfstream*, 6-7 (US natn. Oceanic & Atmospheric Admin.)
74. Richardson, P. L. *J. geophys. Res.* **88**, 4355-4367 (1983).

Fractal growth of copper electrodeposits

R. M. Brady & R. C. Ball

Cavendish Laboratory, University of Cambridge, Madingley Road, Cambridge CB3 0HE, UK

Black dendritic growths of copper were electrodeposited in steady-state diffusion-limited conditions onto an initially point-like cathode. Their spatial extent R was inferred from the increased current and their mass M from the total charge transferred. Fractal behaviour $M \propto R^D$ was observed with $D = 2.43 \pm 0.03$, in good agreement with computer simulations of diffusion-limited aggregation.

IF the accretion of material onto a surface is limited by diffusion onto the surface, deposition occurs preferentially on any protuberances (just as electric field lines concentrate on the convexities of a lightning conductor). In this way any initial roughness grows exponentially—the Mullins-Sekerka (MS) instability—and the deposit rapidly acquires a dendritic quality. Surface properties limit how finely ramified the deposit grows. This instability has been long recognized by materials scientists, with examples ranging from alloy solidification patterns to dendritic crystal growth (see ref. 1 for a review).

Recently, Witten and Sander² introduced a simple computer model in which particles diffuse onto a cluster by random walk, one at a time. This model differs from experimental systems

principally by the absence of any surface tension or crystalline factors and by the very slow rate of reaction; and in this 'diffusion-limited aggregation' (DLA) model the clusters grow immediately dendritic and disorderly. They were shown to be fractal³ in the sense that inside a large cluster the number of particles within a radius R of any given particle was on average proportional to R^D . The index D is the Hausdorff dimensionality⁴ of the deposit and was found by computer simulation in three dimensions by Meakin⁵ to be $D = 2.495 \pm 0.06$. Note that this model does not seem to be applicable to experiments (ref. 6 and D. Weitz, in preparation) where clustering of clusters was allowed and $D = 1.7$ obtained.

Even in the experimental systems where the MS instability is

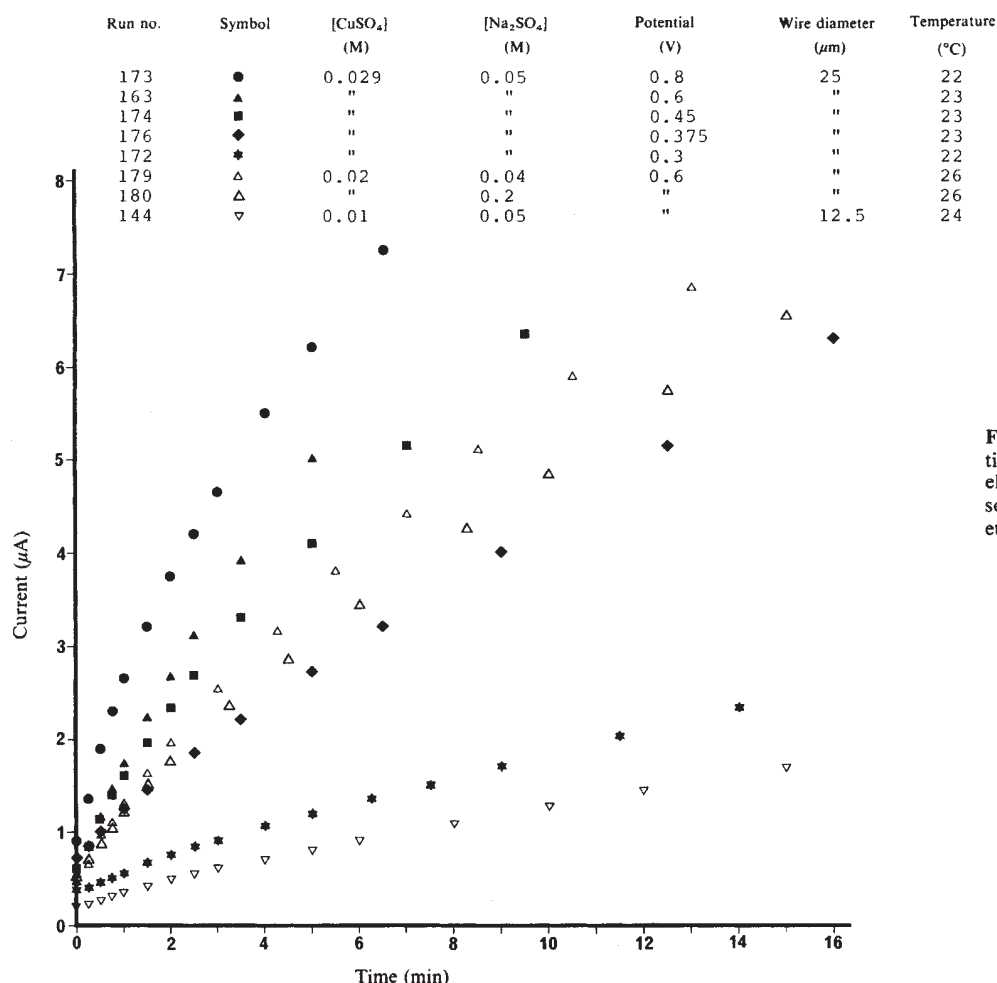


Fig. 1 Increase of current with time, for various potentials and electrolyte concentrations. All the solutions contained 1.4 wt% polyethylene oxide to impede convection.

limited by surface properties, Witten and Sander² suggested that weak noise should ultimately lead to disorder and similar fractal behaviour. Thus, at short length scales there may be local order due to surface tension or crystallographic factors, but at longer length scales they suggested random fluctuations should dominate: in particular, the Hausdorff dimensionality D found by computer simulation should also characterize experimental systems at long length scales.

We now report an experimental measurement of the Hausdorff dimensionality D of copper deposited electrolytically from aqueous solution in diffusion-limited conditions, onto an

initially point-like cathode. The deposits were grown larger than the initial cathode but smaller than any diffusion length in the system. Because the diffusion-limited current onto such a spherical absorber is proportional to its radius (and⁷ not its surface area), the instantaneous current gave an effective overall radius R_s for the deposit. With the mass M of the deposit determined from the total charge transferred, the relationship between M and R_s could then be inferred, and the fractal law $M \propto R_s^D$ verified.

Experiment

Solutions of copper sulphate and sodium sulphate were made up using deionized, distilled water. Polyethylene oxide (C₂H₄O)_n with molecular weight 6×10^6 was added at 1.4 wt%, increasing the viscosity by a factor of 1,000 to ~1 P. The solutions were stored away from light and vigorous stirring was avoided, to prevent damage to the polymer.

In each experiment a sample of the solution was placed inside a hole of diameter 7 mm drilled in a copper block, which acted as the anode. Insulated wire of diameter 12.5 or 25 μm, the end freshly cut with a razor blade, acted as the cathode. The copper deposit would grow in a dendritic cluster around the cut end of the wire which was suspended vertically into the solution. To prevent mechanical movement during the experiment the free length of wire was kept to <7 mm, a vibration-free mounting covered with a bell-jar was used, and at least 5 min were allowed for any movement to die down before the voltage was applied.

The experiments were performed at constant voltage set in the range 0.3–0.8 V, and the current through the cell was measured as a function of time. Initially the interval between readings was 10 s, increasing to several minutes as the current rose more slowly. Typically the experiments were stopped after half an hour.

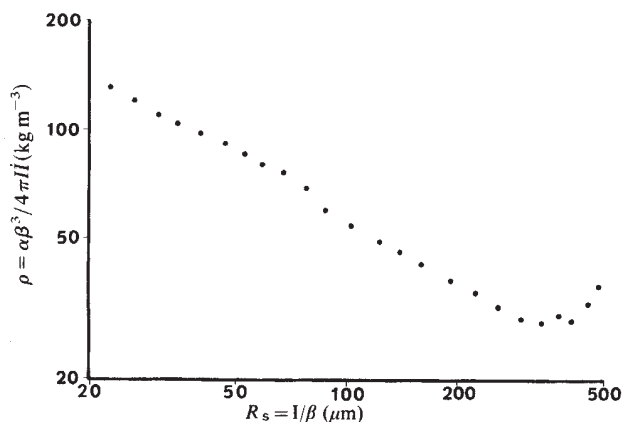


Fig. 2 Logarithmic plot of inferred density as a function of radius, for run 144.

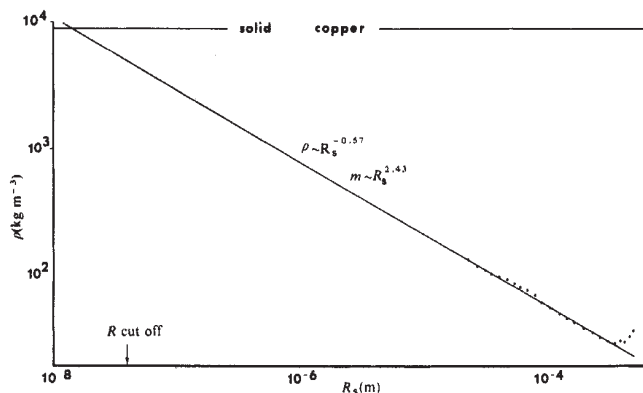


Fig. 3 The data of Fig. 2 plotted on a scale showing the density of solid copper and the radius R_{cutoff} of crystallites observed in electron micrographs. Note the extrapolation (solid line) corresponds to a Hausdorff dimensionality of 2.43.

Figure 1 shows direct plots of current I against time t for various voltages and electrolyte concentrations. It can be seen that the current grows much faster for higher voltages and higher concentrations of copper sulphate, but is relatively insensitive to the sodium sulphate concentration.

It was noted that changing the instantaneous voltage did not change the instantaneous current significantly in our experiments. Any change would have indicated that the reaction was not limited by diffusion of Cu^{2+} ions but was dominated by some other mechanism. The set voltage thus influenced the current-time curves through the morphology of the deposit and not directly through any ohmic or activation limit on the conduction.

Figure 2 shows, with interpretations discussed below, the rate of change of current dI/dt plotted as $-\log I(dI/dt)$ against $\log I$. Plotted in this way the experiment was reproducible to better than 10% except at high currents. Problems at high current and hence large radii included mechanical motion (for example, the wire bending under the weight of the deposit), stirring of the solution, and contact resistances (later improved by applying silver dag).

Results and interpretation

We define an effective radius for the deposits in terms of the diffusion-limited current. Modelling the cathode growth by a perfect spherical absorber of radius R_s , the current⁷ is

$$I = \beta R_s \quad (1)$$

the coefficient β being given by

$$\beta = 4\pi KCq \quad (2)$$

where K is the diffusion coefficient, q the charge, and C the number density far from the absorber of the copper ions. This 'Smoluchovski radius' R_s characterizes where the deposition is taking place, and thus how far out the deposit extends in space. Clearly R_s will be of the same order of magnitude as the radius of gyration, R_g , measured in computer simulations^{2,5}. Indeed, provided the growths are self-similar fractals as the simulations suggest, within the diffusion-limited regime the ratio R_s/R_g will be a constant independent of all parameters.

The number N of copper atoms deposited can be inferred from the charge transferred Q ,

$$N = Q/q = \alpha \int I dt \quad (3)$$

Using equations (1)–(3), the relationship between the radius R_s and the number of atoms N can in principle be observed directly throughout the growth of the deposit. In practice the cathode was initially of finite size so that there would be an offset error

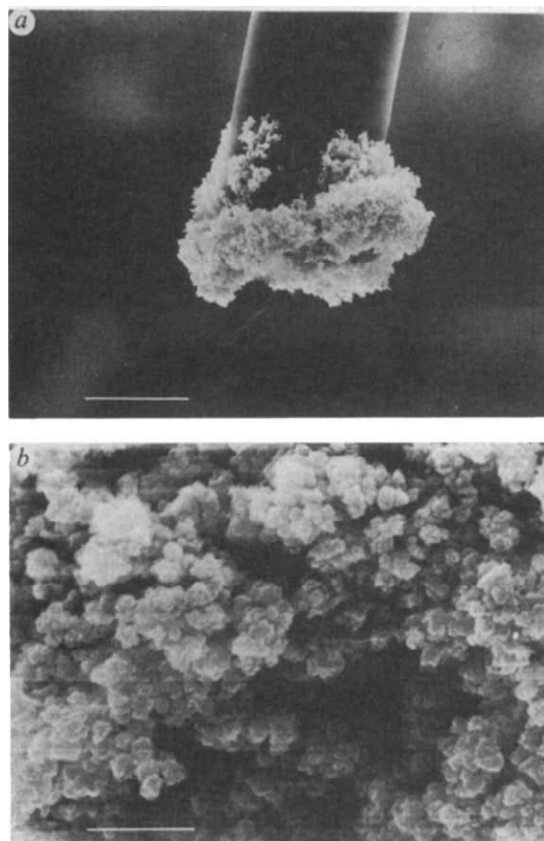


Fig. 4 Electron micrograph of a deposit grown for only 1 min in the absence of polymer. 0.01 M CuSO_4 , 0.05 M Na_2SO_4 , 0.6 V. Scale bars: a, 20 μm ; b, 1 μm .

in the N obtained by integration. It is to avoid this that we have instead plotted dI/dt in Fig. 2.

The quantity

$$\rho = \frac{\beta^3 \alpha}{4\pi I \frac{dI}{dt}} \quad (4)$$

may be interpreted as an effective density of deposition since, using the definition (1) above,

$$dN = 4\pi R_s^2 dR_s \rho \quad (5)$$

Figures 2 and 3 show the density-radius plot inferred from the data shown in Fig. 1. Note the logarithmic straight line $\rho \propto R_s^{-0.57}$ which implies fractal behaviour, $N \propto R^D$ with $D = 2.43$. Writing this result in the form

$$\rho = \rho_{\text{Cu}}(R/a)^{D-3} \quad (6)$$

where ρ_{Cu} is the density of solid copper, the length a characterizes the onset of fractal behaviour. Extrapolating the data in Fig. 3 back to the solid copper density gives an intercept of

$$a = 14 \text{ nm} \quad (7)$$

which compares with the characteristic radius of the dendritic features seen in the electron micrographs (Fig. 4)

$$R_{\text{cutoff}} \approx 40 \text{ nm} \quad (8)$$

The specimen in the micrographs was produced in similar conditions, except that it was grown for only 1 min and with no polymer present so as to minimize damage on removal from the solution.

The experiment was repeated at various electrolyte concentrations as listed in Table 1 together with the Hausdorff dimensionalities D obtained. The values of D show no evidence of systematic variation and taken together give

$$D_{\text{experiment}} = 2.43 \pm 0.03 \quad (9)$$

which compares with the result from DLA computer simulations⁵

$$D_{\text{DLA simulation}} = 2.495 \pm 0.06 \quad (10)$$

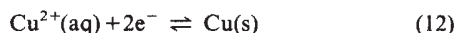
For each run the intercept a was extrapolated using the value $D = 2.43$, and Fig. 5 shows how the reciprocal radius $1/a$ varied with the voltage of the experiment. We have no explanation for the apparent law,

$$1/a \propto V - (0.23 \text{ V}) \quad (11)$$

Note that the intercept a in Fig. 5 does not depend on the concentration of copper sulphate.

Electrochemical considerations

Our interpretation of the experiment presupposes that the net reaction at each electrode was



and that the reaction was limited by diffusion of Cu^{2+} ions in the vicinity of the cathode.

To evolve hydrogen gas at the cathode in the principal experimental conditions (0.01 M CuSO_4 , 0.05 M Na_2SO_4 , neutral pH) requires at least 0.685 V (ref. 8). Below this limit there are no anion oxidations available, and the only remaining chemical difficulty is the role of singly charged Cu^+ ions. In experimental conditions there is an equilibrium ratio in the presence of copper (see ref. 8),

$$[\text{Cu}^+]/[\text{Cu}^{2+}] = 7 \times 10^{-3} \quad (25^\circ\text{C}, 0.01\text{M Cu}^{2+}) \quad (13)$$

and such a concentration of Cu^+ is soluble. With the anode working at low current density (of the order of $1 \mu\text{A cm}^{-2}$), we presume that this equilibrium was maintained and therefore that the current due to Cu^+ ions was negligible.

If stirring of the solution is prevented, the electric fields will be screened and deposition will be limited by diffusion in the neighbourhood of the cathode. The calculated Debye-Huckel screening length for 0.05 M sodium sulphate is $\lambda = 1 \text{ nm}$. (There is in fact a small unscreened voltage of the order of

$$V = kT/e([\text{Cu}^{2+}]_{\text{bulk}} - [\text{Cu}^{2+}])/[\text{Na}_2\text{SO}_4] = 5 \text{ mV} \quad (14)$$

required to maintain neutral charge density as the Cu^{2+} ions adopt a density profile governed by diffusion kinetics.)

Data from the Smithsonian Tables⁹ indicate an ionic diffusion constant for aqueous Cu^{2+} at 24°C of $K = 6.17 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$, with a temperature coefficient of 0.0229 K^{-1} . This implies a value for our parameter β (equation (1)) of

$$\beta = 0.0121 \text{ A m}^{-1} (\pm 5\%) \quad (15)$$

We assume that the polymer had little effect so that the main error in the value for β was the use of dilute solution approximations.

Diffusion lengths

The assumption of Smoluchovski kinetics (equations (1), (2)) requires that the cathode radius R is less than any intrinsic diffusion lengths limiting the scope of a laplace equation for $[\text{Cu}^{2+}]$.

The rate at which the diffusant concentration can relax determines a diffusion length $\xi_i = \sqrt{Kt}$. From equations (1)–(4) the dimensionless ratio $\gamma_i = R/\xi_i$ is of the order of

$$\gamma_i = \sqrt{C/\rho} \quad (16)$$

reaching a maximum value of 0.1 for the largest deposits grown (see Fig. 3) with a concentration of copper sulphate $C = 0.01\text{M}$.

The fluid close to the cathode is depleted of copper ions and is thus less dense by about 2% than the surrounding fluid in

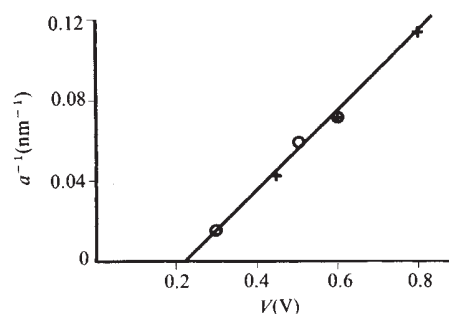


Fig. 5 Variation with voltage of intercept radius a . 0.05 M Na_2SO_4 , 1.4 wt% polyethylene oxide. Concentration of copper sulphate (redetermined by solution conductivity): +, 0.029 M; ○, 0.01 M.

Table 1 Fractal dimension of copper electrodeposited from solution at 0.6 V

Run no.	$[\text{CuSO}_4]$ (M)	$[\text{Na}_2\text{SO}_4]$ (M)	Measured dimension
144	0.01	0.05	2.43
146	0.01	0.05	2.425
150	0.01	0.05	2.465
153	0.01	0.05	2.485
177	0.02	0.04	2.38
178	0.02	0.2	2.44
179	0.02	0.04	2.425
180	0.02	0.2	2.41
Mean dimension			2.433 ± 0.03
Computer prediction ⁵			2.495 ± 0.06

The solutions contained 1.4 wt% polyethylene oxide to increase viscosity.

experimental conditions. Comparing the resulting flux due to convection with that due to diffusion gives a dimensionless parameter

$$\gamma_c = R/\xi_c = mgCR^3/\eta K \quad (17)$$

where mg is the weight of a copper ion and η is the viscosity of the solution. This gives a room temperature value $\gamma_c = 0.02$ for twice the radius of the thinnest wire used ($R = 25 \mu\text{m}$) if no polymer is added. Because $\gamma_c \propto R^3$ this would leave less than one decade of radius before convection became important.

The addition of 1.4 wt% polyethylene oxide raised the overall viscosity by a factor of $\sim 1,000$ without significantly affecting the diffusion of small inorganic ions. The range of diffusion-limited behaviour is thus extended in radius by an order of magnitude and the damping of mechanical disturbances also improved.

Conclusions

The two principal conclusions are that the electrolytic deposit of copper in experimental, diffusion-limited conditions is fractal, and that the Hausdorff dimensionality agrees closely with the simple computer model of diffusion-limited aggregation^{2,5}. This is the first experimental verification of the conjecture by Witten and Sander that such fractal behaviour should occur at long length scales irrespective of local details. The experimental deposits were highly ordered up to order 40 nm, but the Hausdorff dimensionality was measured over the range 25–400 μm . Extrapolating the experimental data and comparing with electron micrograph observations suggests that in fact the fractal behaviour extends over the full range 40–400,000 nm.

There are several possible sources of systematic error in the present experiment. Convection, agitation, contact resistance and resistance and rigidity of the deposit were all problems that we believe were brought under control. Other possible problems

include non-inertness of the polymer, and the presence of any surface-active impurities.

In future work there are four aspects of the experiment which could be improved or varied: the type of measurement made; the electrolyte solution; the geometry; and the physical regime.

That the surface area of the deposit is proportional to its mass could be verified by measuring the capacitance. If the screening length in the solution is short and resistances can be rendered small or corrected for, the capacitance should be proportional to surface area.

The complications associated with the two possible oxidation states of copper might be avoided by, for example, the use of silver electrolytes. Convection might be suppressed without resort to polymer by the use of aqueous Ni^{2+} in the presence of excess CsCl: with these species there is the possibility of arranging that there be no net density change of the solution near the cathode. Other viscosity enhancers might also be tried.

It might be possible to make measurements in a two-dimensional geometry by, for example, using filter paper soaked in the electrolyte. In three-dimensional experiments, the use of microelectrodes would allow measurements to be made down

to smaller length scales, but there could be mechanical problems with such an arrangement. A novel and more stable geometry would be to grow from a 'pinprick' (perhaps made by a spark) in the insulation on a plate: it would be useful to perform the corresponding computer simulation for growth from a point seed into the half space above a plane.

In our experiments the deposits grew slowly compared with the diffusion of ions. It should be possible by using higher electrolyte concentrations to explore the region where the deposits grow more quickly and behaviour is controlled by the diffusion length^{1,10}. An alternative regime leading to fractal deposition might be obtained by stirring the solution until the reaction becomes limited not by diffusion but by ohmic resistance. It would then be the equations of electrostatics which drive the MS instability at long length scales.

R.C.B. and R.M.B. acknowledge Research Fellowships from Gonville and Caius College and from Trinity College, Cambridge respectively. We also thank Union Carbide Ltd for supplying their WSR 301 Polyox, the polymer used in our experiments. Finally we thank Drs J. Agar, M. Archer, J. Deutsch and P. Luckham for valuable advice and discussions.

Received 21 October 1983; accepted 13 March 1984.

1. Langer, J. *Rev. Mod. Phys.* **52**, 1–28 (1980).
2. Witten, T. A. & Sander, L. M. *Phys. Rev. Lett.* **47**, 1400–1403 (1981).
3. Mandelbrot, B. B. *The Fractal Geometry of Nature* (Freeman, San Francisco, 1977).
4. Hausdorff, F. *Math. Ann.* **79**, 157, 179 (1919).
5. Meakin, P. *Phys. Rev. A* **27**, 604–607, 1495–1507 (1983).

6. Forrest, S. R. & Witten, T. A. Jr *J. Phys.* **A12**, L109–L117 (1979).
7. von Smoluchowski, M. *Phys. Z.* **17**, 557–571, 585–599 (1916); *Z. Phys. Chem.* **92**, 129–143 (1917).
8. *Handbook of Chemistry and Physics* 60th edn (CRC Press, 1980).
9. *Smithsonian Physical Tables* 9th edn 398–399 (1954).
10. Nauenberg, M., Richter, R. & Sander, L. M. Preprint NSF ITP 83-60 (1984).

Two protein-binding sites in chromatin implicated in the activation of heat-shock genes

Carl Wu

Laboratory of Biochemistry, Developmental Biochemistry Section, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205, USA

The resistance to exonuclease digestion of two regions of chromatin at the 5' end of heat-shock genes in Drosophila implies they have protein bound to them. The pattern of resistance before and after induction of gene expression suggests that heat-shock genes are activated by the sequential binding of at least two protein factors.

THE continuous array of nucleosomes along the eukaryotic chromatin fibre is punctuated by local discontinuities which are hypersensitive to endonuclease attack. Discovered originally at the origin of replication in the simian virus 40 (SV40) minichromosome^{1–4} and in cellular chromatin encompassing the 5' end of several *Drosophila* heat-shock genes^{5,6}, nuclease hypersensitive sites spanning about 100–300 bp of DNA in chromatin have since been widely observed (see refs 7, 8 for recent reviews). Usually, but not always, the hypersensitive sites are located near the 5' end of genes. Their appearance precedes the onset of transcription and is also tissue- or stage-specific in developmentally regulated genes. The extreme susceptibility to nuclease attack is generally considered to indicate a local opening in chromatin for regulatory proteins and enzymes to enter and bind to target DNA sequences.

I have developed an *in situ* exonuclease III (Exo III) protection technique which maps sequences within nuclease hypersensitive sites in chromatin onto which regulatory proteins might be bound. Short stretches of DNA amidst the 5' hypersensitive sites of the genes for the heat-shock proteins of molecular weight 83,000 and 70,000 (hsp83, hsp70) in *Drosophila* are indeed found to be resistant to exonuclease digestion. The resistant

regions cover the TATA box sequence and an upstream control element.

5' hypersensitivity of the hsp70 gene

Hsp70 is the most abundant of the small group of proteins inducible in *Drosophila* and other organisms by heat shock or other environmental stress⁹. There are five copies of the uninterrupted 2.2 kilobase pair (kb) hsp70 coding sequence, located at two chromosomal loci, 87A and 87C. The hsp70-coding region and about 350 base pairs (bp) of 5' flanking sequences are highly conserved among all five genes^{9–11}.

Fine-structure analysis of the DNase I hypersensitive site at the 5' end of the hsp70 gene gives an initial hint for a resistant region buried within. Nuclear chromatin from noninduced *Drosophila* cells (Schneider line 2, SL2) is digested gently with DNase I. After purification, the DNA is cleaved with a restriction enzyme, *Bam*HI, electrophoresed on a long agarose gel, blotted to nitrocellulose, and hybridized to a ³²P-labelled, probe segment chosen to abut the common *Bam*HI site at position +1,262 in the hsp70 gene sequence; +1 is the transcriptional start (Fig. 1). This effectively labels subfragments extending from that restriction site to the DNase I break made in the initial digestion;

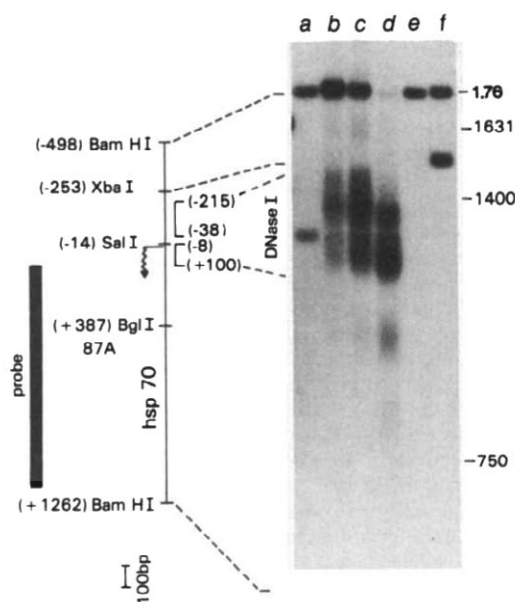


Fig. 1 DNase I hypersensitivity at the 5' end of the noninduced hsp70 gene. A partial restriction map of one of the two hsp70 genes at chromosomal locus 87A is shown¹¹. The start site (+1) and direction of transcription is indicated by the arrow.

Methods: Nuclei from *Drosophila* SL2 cells were isolated and digested with DNase I at 6, 8 and 4 U ml⁻¹ (a separate reaction) respectively as described previously⁵ (lanes b–d). Purified DNA (10 µg per lane) was restricted with *Bam*HI, electrophoresed on a 40 cm long, 1.4% agarose Tris-acetate EDTA (TAE) gel, blotted, hybridized to a ³²P-labelled, cloned probe fragment (solid bar) and autoradiographed as described previously⁵. For markers, SL2 DNA was restricted partially with *Sal*I, *Bam*HI and *Xba*I respectively, followed by complete *Bam*HI restriction and processing as above (lanes a, e, f).

thus the DNase I break points can be positioned by simple length measurement (the indirect end-labelling method)^{5,12}. The probe used detects the *Bam*HI fragments and cleaved subfragments from all five hsp70 genes, although only the 1.76-kb *Bam*HI fragment corresponding to one gene is included in Fig. 1; the larger *Bam*HI fragments have been cropped away⁵. As noted before, the DNase I cleavages in chromatin span a broad region around the 5' end of the hsp70 gene, although some suppression and enhancement is now clearly evident (lanes b–d). For precise mapping, genomic DNA restricted additionally at landmark sites are electrophoresed in adjacent lanes (lanes a, f), or are co-electrophoresed with the samples (data not shown). Thus the hsp70 sequences hypersensitive to DNase I in chromatin extend from positions +100 to –8 and from –38 to –215, with a peak at –93. The 30-bp region in between, from –8 to –38, is relatively resistant. These measurements are precise to 5–10 bp. The complex digestion pattern could be due to different contributions from each of the five hsp70 genes. However, it is qualitatively unchanged when the genes at either chromosomal locus are removed with specific restriction cleavage (data not shown); thus the average pattern is likely to represent each hsp70 gene. The site from –8 to –38 that is resistant to partial cleavage suggests bound nonhistone protein. For more conclusive evidence, a new test has been devised which can visualize the resistant fragments positively after limit nuclease digestion.

Mapping Exo III-resistant sites

One way to locate the borders of protein-bound sites on a piece of DNA is to nibble in from either end with an exonuclease, usually *Escherichia coli* exonuclease III¹³, until further passage of the enzyme is blocked, and to measure the length of the

resistant DNA strand in such a limit digest. This approach was used successfully to localize protein-binding sites in reconstituted complexes of DNA with purified *E. coli* RNA polymerase, λ integrase and SV40 T antigen, and to analyse the disposition of DNA in native and reconstituted nucleosomes^{14–26}. Here I combine the use of Exo III with the indirect end-labelling method to detect the presence of specific barriers against Exo III digestion in chromatin.

As Fig. 2A shows, an initial cut is made within a hypersensitive site by a (partial) endonuclease reaction on isolated nuclei. This creates a free end from which Exo III can digest 3' to 5' along the DNA until a specific block is encountered in the limit. The DNA is purified and the single-stranded tails are trimmed with *S*₁ nuclease. After further cleavage with flanking restriction enzymes it is possible to determine the relative start and stop positions of Exo III by indirect end-labelling the subfragments produced from endo- and exonucleolytic cleavages.

For the noninduced hsp70 gene, the initial cuts can be made by each of four different endonucleases: DNase I, micrococcal nuclease, a Ca²⁺–Mg²⁺ stimulated endogenous nuclease, and a restriction enzyme *Xho*I which cleaves at position –194, inside the distal boundary of the DNase I hypersensitive site (Fig. 2B). Exo III is added in increasing amounts simultaneously except for micrococcal nuclease, which is inactivated with EGTA without affecting Exo III added subsequently. The purified and *S*₁-trimmed DNA is restricted with *Bam*HI and *Xba*I, and the relative positions of the endo- and exonuclease cleavages are located by indirect end-labelling.

The complex pattern of 5' hypersensitivity is again observed when only the initial cuts are made with no added Exo III (Fig. 2B, lanes b, j). However, with the introduction of Exo III, the fragments upstream of the 30-bp, resistant site coalesce sharply to a discrete position on its distal edge, no matter which enzyme is used to make the initial cut. The discrete barrier clearly persists even when Exo III activity is increased roughly 100-fold (lane i). When the first cut is made by *Xho*I at position –194, Exo III nibbles almost quantitatively from that site to the same discrete stop. The distance travelled by Exo III from the *Xho*I site can be determined precisely (to within 5 bp) by measuring the relative difference in fragment lengths between the two subfragments, using DNA markers in adjacent gel lanes, or mixed in together with the samples (see Fig. 3). Thus the block in the noninduced hsp70 gene is located at position –40.

No Exo III resistance on protein-free DNA

Two potential artefacts can compromise the technique. The discrete block observed could be due to a specific cut made by *S*₁ nuclease on the purified DNA during the trimming step, or to a peculiar DNA sequence which blocks the passage of the enzyme by virtue of primary or secondary DNA structure alone. In a control experiment using protein-free DNA as substrate (Fig. 2C), no specific cuts are shown to be made by *S*₁ nuclease on free DNA at the position of blockage in chromatin, nor at any other position in the region analysed (lane c). Also, no impediments against Exo III digestion due to the DNA sequence alone are observed (lane d). Hence, the resistance to Exo III digestion is due to nucleoprotein structure in chromatin.

Heat shock

Does the pattern of Exo III digestion from the *Xho* I cut at –194 change during heat shock? Although the efficiency of restriction is somewhat reduced in the induced hsp70 gene, the digestion pattern is qualitatively different from the noninduced pattern (Fig. 3). Instead of a discrete stop at –40, the enzyme stutters from position –108 to –40, indicating that a comparatively weak barrier (or series of barriers) is encountered, starting at –108. The different patterns of resistance in the normal and the induced genes are found to be similar at both chromosomal loci, using additional restriction cleavage which effectively removes the genes at either locus (data not shown); thus the average pattern is likely to represent each individual hsp70 gene.

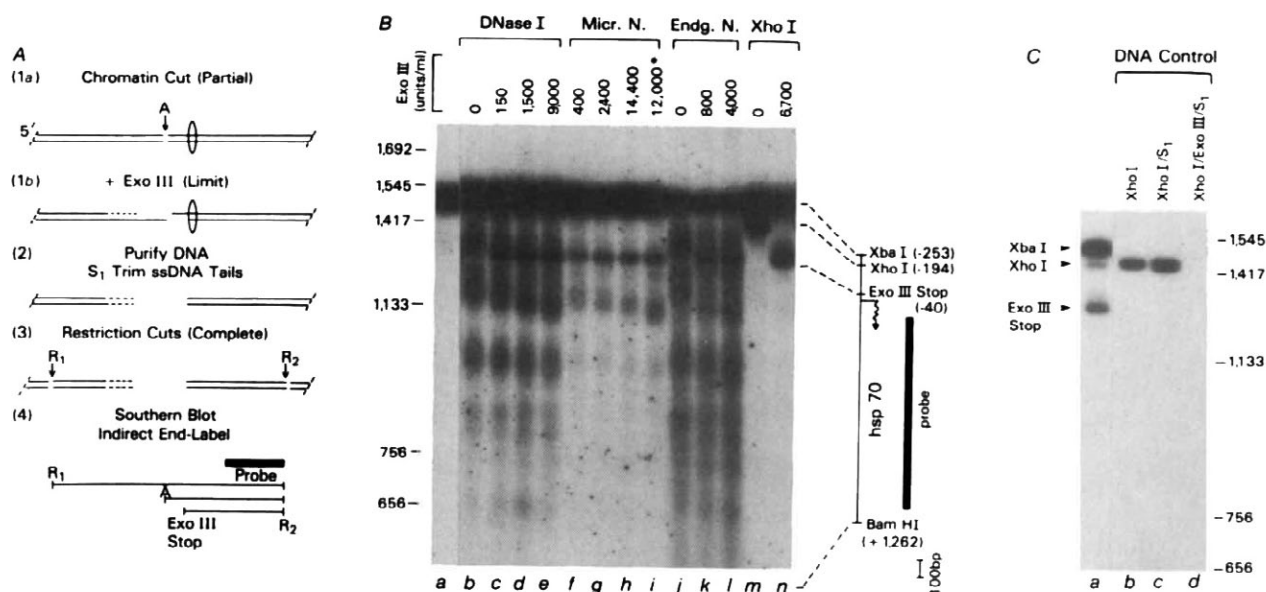


Fig. 2 **A**, The Exo III protection technique. A hypersensitive site in chromatin is drawn as a region of DNA with a hypothetical protein bound to it. **B**, Exo III resistance in chromatin in the noninduced hsp70 gene. A partial restriction map of the hsp70 gene is shown¹¹. Lane *a*, No enzyme control. SL2 nuclei were mock-incubated in digestion buffer. Lanes *b-e*, SL2 nuclei were digested with DNase I, 10 U ml⁻¹ and Exo III for 11 min at 30 °C. Lanes *f-i*, 0-18 h embryonic nuclei were digested with micrococcal nuclease, 9 U ml⁻¹, 3 min, 25 °C. The enzyme was inactivated with EGTA to 0.5 mM, Exo III was introduced and the digestion continued at 30 °C for 7 min (lanes *f-h*) or for 30 min (lane *i*)*. Lanes *j-l*, SL2 nuclei were digested with an endogenous nuclease stimulated by digestion in buffer plus 1 mM CaCl₂, at 37 °C for 10 min. CaCl₂ was chelated with EGTA to 1.5 mM, Exo III was added and digestion continued at 37 °C for 15 min. Lanes *m, n*, SL2 nuclei were digested with *Xho*I at 2,000 U ml⁻¹ and Exo III for 10 min, 30 °C. **C**, S₁ nuclease and Exo III reactions on protein-free DNA. **Methods:** **B**, Isolation of nuclei: SL2 cells were disrupted at 0-4 °C in a Dounce homogenizer in nuclear buffer (60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 15 mM Tris-HCl pH 7.4, 0.5 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.3 M sucrose). Unbroken cells were pelleted at 1,600g for 45 s. The supernatant was centrifuged through 1 vol of nuclear buffer-1.7 M sucrose (stirred interface), at 17,000g (average) for 15 min in a swinging bucket rotor. Purified nuclei were resuspended for digestion in nuclear buffer-5% glycerol at 5 × 10⁸ nuclei ml⁻¹. Nuclei from *Drosophila melanogaster* embryos (Oregon R, P2 strain) were prepared after dechorionating in 50% Chlorox, 0.02% Triton X-100 for 3 min at room temperature, by disruption in a Dounce homogenizer in nuclear buffer at 0-4 °C. The homogenate was filtered through a crude paper filter and the filtrate was centrifuged at 1,600g for 90 s. The supernatant was centrifuged at 3,700g (average) for 5 min in a swinging bucket rotor to pellet crude nuclei. The pellet was resuspended in nuclear buffer, and further centrifuged through nuclear buffer-1.7 M sucrose as above. Purified embryonic nuclei were resuspended for digestion in nuclear buffer-5% glycerol at 1 × 10⁹ nuclei ml⁻¹. Nuclease digestion: Nuclear buffer-5% glycerol was used as the standard buffer for all digestions except those involving micrococcal nuclease, which included 0.4 mM CaCl₂. Nuclei were digested with the primary cleavage enzyme plus Exo III (New England Biolabs or Boehringer Mannheim). Digestions were terminated as described previously⁵ and nucleic acids were purified by overnight proteinase K digestion, 50 µg ml⁻¹, 37 °C, and by repeated organic extractions followed by ethanol precipitation⁶. S₁ nuclease trimming: Reactions were performed in 40 mM NaOAc, pH 4.5, 1 mM ZnSO₄ and 250 mM NaCl using 4,000 U ml⁻¹ S₁ nuclease (Boehringer Mannheim) at 30 °C for 15 min. The DNA concentration was typically ~0.2 mg ml⁻¹. The reaction was terminated by the addition of EDTA to 5 mM and Tris-HCl pH 8.0 to 50 mM. Nucleic acids were purified by organic extraction and ethanol precipitation. Restriction digests and gel electrophoresis: DNA samples (~5 µg per lane) were secondarily restricted to completion using *Bam*HI+*Xba*I, together with a trace of RNase A and RNase TI. DNA was electrophoresed on a 40 cm long, 1.4% agarose TAE gel at room temperature for ~35 h. The gel was treated in 0.1 M HCl for 15 min before the DNA was denatured, neutralized and blotted, as described previously⁵ except that the blotting solution was 20x SSC, 1 M NH₄OAc (1x SSC is 0.15 M NaCl, 0.015 M Na citrate pH 7.0). The filter was rinsed in 1/2 × blotting solution, dried, baked, prehybridized for 4-8 h at 65 °C and hybridized as described previously⁵ without dextran sulphate for 12 h at 65 °C in a slit chamber to a recombinant plasmid labelled with ³²P by nick-translation to a specific activity of ~4 × 10⁸ c.p.m. per µg DNA. The solid bar indicates the region of the hsp70 gene in the plasmid. The filter was washed twice at 65 °C in 2 × SSC, 0.5% SDS and three times in 0.1 × SSC, 0.1% SDS, 65 °C; each wash was for 15 min with agitation. The filter was exposed without intensification. **C**, 20 µg purified SL2 DNA was restricted with *Xho*I in 25 µl of 100 mM NaCl, 6 mM MgCl₂, 10 mM Tris pH 7.4. After the reaction 25 µl of nuclear buffer was added and 5 µl of the mixture was removed for secondary *Bam*HI restriction (lane *b*). Lane *c*, DNA was purified by phenol extraction from 22.5 µl mixture. The phenol was removed by ether extraction, the DNA treated with 2,500 U ml⁻¹ S₁ nuclease as in Fig. 2b legend, purified, and digested with *Bam*HI. Lane *d*, the last 22.5 µl of mixture were digested with 4,000 U ml⁻¹ Exo III at 30 °C for 10 min. DNA was purified, S₁ trimmed, repurified and restricted with *Bam*HI. Samples *b-d* were electrophoresed, blotted and hybridized to hsp70 probe as in **B** legend, along with a typical *Xho*I+Exo III reaction on chromatin for comparison (lane *a*).

Other side of barrier

The other side of the barrier to Exo III in the normal and induced hsp70 genes is mapped using *Pvu*II to make the initial cut; the enzyme cleaves at position +65, inside the proximal boundary of the DNase I hypersensitive site (Fig. 4). The hybridization probe labels the 3.56-kb fragment containing one hsp70 gene, but is also weakly homologous to other *Bgl*II fragments; these do not obscure the analysis of the hsp70 subfragments. In both normal and induced samples, a discrete barrier which maps to position -12 is observed (Fig. 4, lanes *c, d, f, g*). A control experiment using protein-free DNA as substrate indicates that this barrier is due neither to artefactual S₁ cleavage nor to the DNA sequence alone (data not shown).

An endogenous exonuclease activity is also observed here, in the reactions without Exo III, but this activity is blocked at essentially the same position (Fig. 4, lanes *b, e*). Taking into account the uneven amounts of DNA loaded, proportionately more of the *Bgl*II-*Pvu*II fragment is chased into the subfrag-

ment, by addition of Exo III. The endogenous activity nibbles predominantly in a single-stranded manner since *Pvu*II restriction of chromatin without subsequent S₁ nuclease reaction gives a fragment which co-migrates with a whole *Bgl*II-*Pvu*II fragment (lane *a*). As expected from previous work²⁷, the sensitivity of a *Pvu*II site at +1,034 in the hsp70-coding region is increased during induction, but no discrete subfragments due to exonuclease activity emerge from this site.

In summary, convergent Exo III digestion from cuts on each flank of the DNase I hypersensitive site reveals strong blockage at positions -40 and -12 respectively, and delimits a 28-bp, resistant site (site I) for the noninduced hsp70 gene. This agrees well with the earlier mapping of the 30-bp, DNase I resistant site from -38 to -8. For the induced hsp70 gene, strong blockage again at -12 suggests retention of site I. In addition, the diffuse blocks from -108 to -40 reveal a new site of weaker resistance (site II), the distal border of which lies at -108; its proximal border cannot be mapped from the other side because the enzyme is unable to penetrate site I.

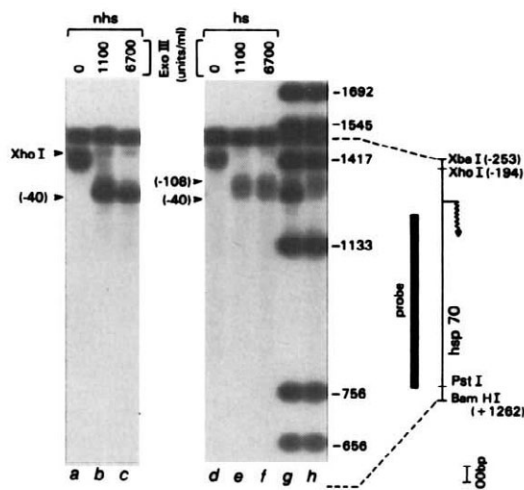


Fig. 3 Exo III digestion from the *Xho*I cut at position -194 in normal and induced hsp70 gene chromatin. Isolated nuclei from 0–18 h old, nonshocked embryos (lanes a–c) and from embryos heat-shocked for 15 min at 36 °C (lanes d–f), were digested for 10 min at 30 °C with *Xho*I at 2,000 U ml⁻¹ plus Exo III as indicated. Purified DNA samples were digested with *S*₁ nuclease, restricted secondarily with *Bam*HI + *Xba*I, electrophoresed, blotted and hybridized with the hsp70 gene probe as described in Fig. 2B legend. The Exo III stop positions in base pairs upstream from the mRNA start (+1) are indicated beside the arrowheads. Lanes g and h are the same samples as in b and e spiked with pBR322 marker fragments, ~60 pg each (pBR322 restricted with *Bam*/PvuII; *Bam*/Pst; *Eco*RI/*Sal*I/PvuII/PstI).

Exo III resistance on hsp83 gene

The single-copy gene encoding hsp83 resides at chromosomal locus 63BC and consists of a 3.7-kb transcription unit containing two exons separated by a 1.1-kb intron^{28–31}. I have previously mapped a cluster of three DNase I hypersensitive sites, one of which encompasses the 5' terminus of the hsp83 gene³. The Exo III digestion pattern from a *Eco*RI cut at position -166 is shown in Fig. 5A. In noninduced *Drosophila* embryos, the principal subfragment among the heterogeneous collection produced by the endogenous exonuclease and by Exo III locates a barrier at position -39 (Fig. 5A, lanes b, c). On heat induction, this barrier shifts sharply to position -86 (lanes d, e).

The other side of the barriers are located by using exonuclease to nibble upstream from a *Rsa*I cut at position +20 (Fig. 5B). In noninduced *Drosophila* embryos, the principal subfragment produced by the endogenous exonuclease locates a weak barrier at position -17 (Fig. 5B, lanes b, c). Addition of Exo III further degrades that subfragment; there is a minor pause at -61 (lanes d, e). In heat-shocked embryos, two sharply discrete subfragments, one chasing into the other, are produced by the endogenous exonuclease and by Exo III (Fig. 5B, lanes f, g, h, i), locating two barriers at positions -17 and -50 respectively. No resistant sites are found in the controls using free DNA (data not shown). The relatively lower yield of cleaved subfragments in noninduced samples in Fig. 5 is primarily due to a less efficient initial restriction cut.

In summary, the resistance to convergent exonuclease digestion at -39 and -17 delimits a relatively weak, 22-bp resistant site (site I) for the noninduced hsp83 gene. For the induced gene, blockage again at -17 suggests the retention of site I. In addition, a 36-bp resistant site from -86 to -50 is revealed (site II). The distal border of site I cannot be mapped here because exonuclease is unable to penetrate site II. No highly resistant sites are found within the other two DNase I hypersensitive sites clustered near the 5' end of the hsp83 gene (data not shown).

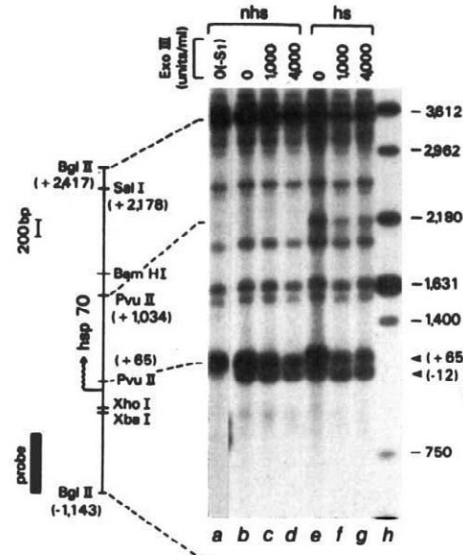


Fig. 4 Exo III digestion from the *Pvu*II cut at position +65 in normal and induced hsp70 gene chromatin. A partial restriction map of the first of the two hsp70 genes in tandem at chromosomal locus 87C is shown¹¹. Isolated nuclei from nonshocked SL2 cells (lanes b–d) and from cells heat shocked for 15 min at 36 °C (lanes e–g) were digested for 40 min at 37 °C with 600 U ml⁻¹ *Pvu*II plus Exo III as indicated. DNA was purified, trimmed with *S*₁ nuclease, repurified, restricted with *Bgl*II, electrophoresed on a 30 cm 1.2% agarose gel, blotted and hybridized with the cloned probe (solid bar) as described in Fig. 2B legend. An arrowhead indicates the Exo III (and endogenous nuclease) stop position. Similar results are obtained using normal and heat-shocked embryos. Lane a is a *Pvu*II digest of nonshocked SL2 nuclei at 400 U ml⁻¹, 30 °C, 20 min; the purified DNA was restricted with *Bgl*II without prior *S*₁-nuclease treatment, and processed as above. Lane h contains pBR322 markers as in ref. 5.

TATA and upstream sequences

The resistant sites are aligned over the nucleotide sequences²⁹ upstream of the hsp83 and hsp70 transcriptional starts in Fig. 6A. They lie within the 5' flanking region involved in the regulation of these genes^{32–36}. Strikingly, for both hsp83 and hsp70 genes, site I surrounds the TATA box sequence which is indispensable for accurate transcriptional starts³⁷. For the induced hsp83 gene, site II covers the upstream consensus sequence CTNGAANNTTCNAG that is required for heat-inducible expression in heterologous transient assays^{33,35}. This upstream element is also contained within the more diffuse site II in the induced hsp70 gene, although the site does extend an additional 45 bp upstream. Curiously, site I is strong in the hsp70 gene whereas site II is strong in the hsp83 gene.

Site II does directly cover the consensus sequence in the induced hsp83 gene. Before induction, two *Xba*I sites at -74 and -64, essentially coincident with the upstream element, are hypersensitive to *Xba*I cleavage in chromatin when compared with a site at +484 within the hsp83 transcription unit. After induction, the upstream *Xba*I sites are almost totally resistant, whereas the latter is more sensitive (Fig. 5B, lanes j, k). A rough analysis (not shown) indicates that sites I and II in the hsp83 gene are also resistant to DNase I digestion.

Discussion

The resistant sites surrounding the TATA box sequence and the upstream control element cover 22 bp or 28 bp and 36 or <68 bp respectively, depending on the gene. This range is considerably less than the 146 bp of DNA resistant to enzyme

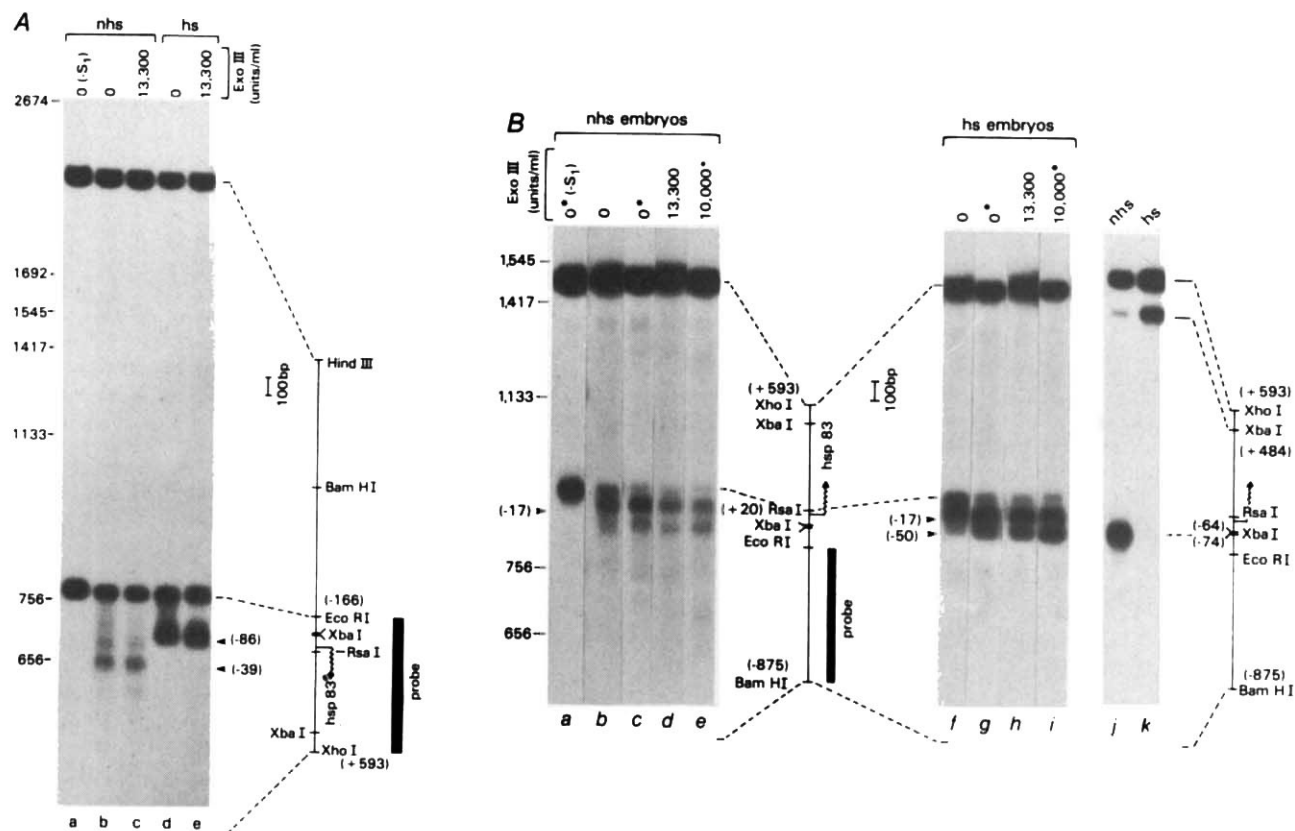


Fig. 5 **A**, Exo III digestion from the *EcoRI* cut at position -166 in normal and induced hsp83 gene chromatin. A partial restriction map of the hsp83 gene region is shown^{28,30}. Isolated nuclei from normal (lanes a-c) and heat-shocked (lanes d, e) 2-8 h-old embryos were digested with *EcoRI* at 4,200 U ml⁻¹ plus Exo III as indicated for 7 min at 30 °C. The purified DNA was trimmed with S₁ nuclease (except in lane a), repurified, restricted with *XhoI*+*HindIII*, electrophoresed on a 40-cm long, 1.4% 50 mM Tris-borate EDTA pH 8.3 (TBE) gel, blotted and hybridized with the cloned probe (solid bar) as described in Fig. 2B legend. Arrowheads indicate the stop positions of Exo III and the endogenous exonuclease. **B**, Exo III digestion from the *RsaI* cut at position +20 in normal and induced hsp83 gene chromatin. Isolated nuclei from normal (lanes a-e) and heat-shocked (lanes f-i) 2-8-h-old embryos were digested with 1,500 U ml⁻¹ *RsaI* plus Exo III as indicated, for 15 min at 35 °C (lanes a, c, e, g, i)* or with 1,700 U ml⁻¹ *RsaI* plus Exo III as indicated for 7 min at 30 °C (lanes b, d, f, h). The purified DNA was trimmed with S₁ nuclease (except in lane a), repurified, restricted with *BamHI*+*XhoI*, electrophoresed on a 1.4% 50 mM TBE gel, blotted and hybridized with the cloned probe (solid bar) as described in Fig. 2B legend. Lanes j, k, *XbaI* cleavage of hsp 83 chromatin. Isolated nuclei from normal (lane j) and heat-shocked (lane k) 2-8-hr-old embryos were digested with 1,400 U ml⁻¹ *XbaI* at 35 °C for 15 min. The purified DNA was restricted with *BamHI*+*XhoI*, electrophoresed beside the above samples and processed similarly.

digestion in the nucleosome core particle³⁸⁻⁴⁰, but is similar in size to the fragments resistant to digestion when prokaryotic activator and repressor proteins are bound to DNA⁴¹. We suggest that the resistance is due to at least two nonhistone proteins which bind specifically to duplex DNA in the TATA box region (TATA-binding protein(s), TAB), and the upstream heat-shock control region (heat-shock activator protein(s), HAP) respectively (Fig. 6b). (Since Exo III is also unable to digest single-stranded DNA, the resistance to cleavage could conceivably occur by a melting of the sequences, but this is improbable because the sites are resistant to DNase I cleavage too.)

We conjecture that the binding of TAB establishes the transcriptional potential of heat-shock genes in (post-blastoderm) embryos. This potential is realized during heat-shock activation by the additional binding of HAP. The fact that site II only appears in the active gene argues that HAP is a positive activator of transcription; a repressor should have had the reverse effect. Others have also argued for positive regulation in the sense that deletions in the upstream element reduce transcription during heat shock rather than cause constitutive expression^{33,34}. Only after TAB and HAP are bound might transcription be initiated by RNA polymerase II. The significance of protein-protein contacts in the binding of polymerase II to eukaryotic promoters has been argued⁴².

The displacement of a nucleosome from the TATA box region by TAB, combined with the perturbation of higher order con-

tacts between neighbouring nucleosomes in the native chromatin fibre, could be sufficient to create nuclease hypersensitivity over the adjacent sequences, its relative intensity and spread being influenced by the local sequence order. Such a displacement could occur during transient reassembly of chromatin during DNA replication.

Is the TATA box sequence in all genes which possess it bound to TAB in chromatin? This seems unlikely in view of other work on developmentally regulated genes which suggest a nucleosomal arrangement over promoter sequences in all but the expressing tissue. Although no obvious additional homologies have been generally found within 10 bp around the TATA box³⁷, Holmgren *et al.*²⁹ have pointed out an eight base homology (A/G)C(C/A)GGCGC immediately following the TATA box in the heat-shock gene family, which, curiously enough, is also present at the same locations in the *Drosophila* histone H4 and alcohol dehydrogenase genes (noted in ref. 33) but not in other *Drosophila* genes. All TATA binding functions could be governed by a small group of TAB proteins, each of which is able to bind to a 'TATA(+)' consensus sequence common to one or more gene families. Davidson *et al.*⁴³ have recently reported a Hela cell factor which is necessary for *in vitro* transcription from conalbumin and adenovirus late promoters, which binds to the TATA box region in the absence of polymerase II and forms a stable pre-initiation complex. It is interesting to speculate that TAB may fall into this class of transcription factors.

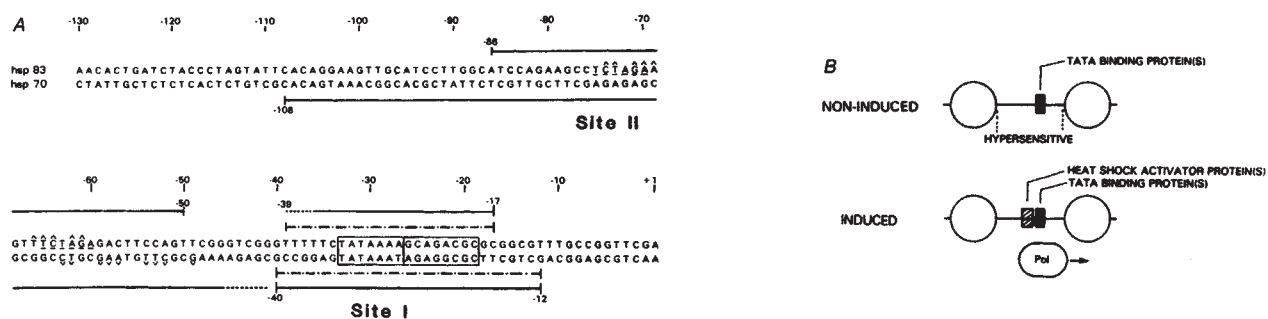


Fig. 6 **A**, Exonuclease resistant sites aligned over nucleotide sequences upstream of the 5' ends of the hsp83 and hsp70 genes. The sequences are copied from Holmgren *et al.*²⁹. The initiation site of transcription of both genes is shown as +1. Boxed sequences indicate homologies shared between these and other *Drosophila* heat-shock genes²⁹. Capped nucleotides indicate the consensus sequence that is required for heat induction³⁵. Underlined nucleotides indicate the two *Xba*I sites upstream of the hsp83 gene. Lines above and below the sequences are resistant nucleotides in hsp83 and hsp70 gene chromatin respectively. ---, Resistant nucleotides in the noninduced genes; —, resistant nucleotides in the induced genes. Boundaries of the resistant regions are precise to five base pairs. Undeterminable boundaries are indicated by dots following the solid lines. **B**, A model for the heat-shock promoter region in chromatin during normal and induced states. Circles represent nucleosomes; their positions have not been determined. Pol refers to RNA polymerase II.

Our simple model does not attempt to account for the more subtle nuances of heat-shock gene expression, such as the developmental control which is superimposed on the hsp83, hsp28 and hsp26 genes in *Drosophila* ovarian nurse cells⁴⁴. We hope it will be useful as a framework for comparison with the rest of the heat-shock gene family and for defining the minimal components essential for heat-shock gene expression. Aspects of the model are generally applicable to the regulation of other eukaryotic genes.

This work has focused on the localization of barriers against convergent Exo III nibbling from cuts on either side of a nuclease hypersensitive site. Divergent cleavage, presumably into adjacent nucleosomal DNA, might reveal the positions of neighbouring nucleosome core particles. We have not extended our analysis into this area, except in one example averaged over the five heat-shock genes, and this result appears to argue in favour of a statistical⁴³ location of these nucleosomes (Fig. 2B). A more detailed analysis is required to address this question properly.

The Exo III protection technique could be used for mapping other protein-binding sites in chromatin. Clean results are

obtained especially when a single-cut restriction enzyme is used to make the initial cleavage in the hypersensitive region. We have found empirically that mild conditions for restriction and Exo III cleavage (30 °C, 10–15 min) using several thousand units per ml of restriction enzyme can result in cleavage of more than 50% of the molecules at a particular hypersensitive site, of which roughly 50–100%, depending on the site, can be digested further by the endogenous exonuclease or Exo III. Thus, the resistant sites occur in a significant fraction of the overall population. For ultimate precision in site mapping, the exonuclease resistant fragments can be separated on sequencing gels (without prior *S*₁ trimming), and the appropriate strand labelled with a single-stranded probe after blotting to nylon membranes according to a new protocol⁴⁶.

I thank R. Morimoto, R. Holmgren, R. Blackman and M. Meselson for gifts of cloned heat-shock genes, and for communicating unpublished sequences, and J. Lis for the latter. I thank my colleagues for helpful suggestions, M. Singer for a critical review of the manuscript, T. Paisley for technical assistance and B. Wood for maintaining our *Drosophila* colony.

Received 12 December 1983; accepted 23 February 1984.

- Varshavsky, A. J., Sundin, O. H. & Bohn, M. J. *Nucleic Acids Res.* **5**, 3469–3478 (1978).
- Varshavsky, A. J., Sundin, O. H. & Bohn, M. J. *Cell* **16**, 453–466 (1979).
- Scott, W. A. & Wigmore, D. J. *Cell* **15**, 1511–1518 (1978).
- Waldeck, W., Fohring, B., Chowdhury, K., Gruss, P. & Sauer, G. *Proc. natn. Acad. Sci. U.S.A.* **12**, 5964–5968 (1978).
- Wu, C. *Nature* **286**, 854–860 (1980).
- Wu, C., Bingham, P. M., Livak, K. J., Holmgren, R. & Elgin, S. C. R. *Cell* **16**, 797–806 (1979).
- Elgin, S. C. R. *Cell* **27**, 413–415 (1981).
- Weisbrod, S. *Nature* **297**, 289–295 (1982).
- Schlesinger, M. J., Ashburner, M. & Tissieres, A. (eds) *Heat Shock from Bacteria to Man* (Cold Spring Harbor Laboratories, New York, 1982).
- Mason, P. J., Torok, I., Kiss, I., Karch, F. & Udvardy, A. J. *molec. Biol.* **156**, 21–35 (1982).
- Karch, F., Torok, I. & Tissieres, A. *J. molec. Biol.* **148**, 219–230 (1981).
- Nedospasov, S. A. & Georgiev, G. P. *Biochem. biophys. Res. Commun.* **92**, 532–539 (1980).
- Rogers, S. G. & Weiss, B. *Meth. Enzym.* **65**, 201–210 (1980).
- Ptashne, M. *et al. Science* **194**, 156–161 (1976).
- Siebenlist, U., Simpson, R. B. & Gilbert, W. *Cell* **20**, 269–281 (1980).
- Schreier, P. H., Davies, R. W. & Kotewicz, M. L. *FEBS Lett.* **109**, 159–163 (1980).
- Shalloway, D. T., Kleinberger, T. & Livingston, D. M. *Cell* **20**, 411–422 (1980).
- Chan, P. T. & Lebowitz, J. *Nucleic Acids Res.* **11**, 1099–1116 (1983).
- Riley, D. & Weintraub, H. *Cell* **13**, 281–293 (1978).
- Prunell, A. & Kornberg, R. D. *Cold Spring Harb. Symp. quant. Biol.* **42**, 103–108 (1977).
- Igo-Kemenes, T., Omori, A. & Zachau, H. G. *Nucleic Acids Res.* **8**, 5377–5390 (1980).
- Horz, W. & Zachau, H. G. *J. molec. Biol.* **144**, 305–327 (1980).

- Prunell, A. & Kornberg, R. D. *J. molec. Biol.* **154**, 515–523 (1982).
- Strauss, F. & Prunell, A. *Nucleic Acids Res.* **10**, 2275–2293 (1982).
- Zhang, X.-Y., Fittler, F. & Horz, W. *Nucleic Acids Res.* **11**, 4287–4306 (1983).
- Chao, M. V., Gralla, J. & Martinson, H. G. *Biochemistry* **18**, 1068–1074 (1979).
- Wu, C., Wong, Y. C. & Elgin, S. C. R. *Cell* **16**, 807–814 (1979).
- Holmgren, R., Livak, K., Morimoto, R., Freund, R. & Meselson, M. *Cell* **18**, 1359–1370 (1979).
- Holmgren, R., Corces, V., Morimoto, R., Blackman, R. & Meselson, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3775–3778 (1981).
- O'Connor, D. & Lis, J. T. *Nucleic Acids Res.* **9**, 5075–5092 (1981).
- Hackett, R. W. & Lis, J. T. *Nucleic Acids Res.* **11**, 7011–7030 (1983).
- Corces, V., Pellicier, A., Axel, R. & Meselson, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7038–7042 (1981).
- Pelham, H. R. B. *Cell* **30**, 517–528 (1982).
- Mirault, M. E., Southgate, R. & Delwart, E. *EMBO J.* **1**, 1279–1285 (1982).
- Pelham, H. R. B. & Bienz, M. *EMBO J.* **1**, 1473–1477 (1982).
- Bienz, M. & Pelham, H. R. B. *EMBO J.* **1**, 1583–1588 (1982).
- Breathnach, R. & Chambon, P. A. *Rev. Biochem.* **50**, 349–383 (1981).
- Kornberg, R. D. A. *Rev. Biochem.* **46**, 931–950 (1977).
- McGhee, J. D. & Felsenfeld, G. A. *Rev. Biochem.* **49**, 1115–1156 (1980).
- Igo-Kemenes, T., Horz, W. & Zachau, H. G. A. *Rev. Biochem.* **51**, 89–121 (1982).
- Goldberger, R. (ed.) *Biological Regulation and Development* Vol. 1 (Plenum New York, 1979).
- Travers, A. *Nature* **303**, 755 (1983).
- Davidson, B. L., Egly, J. M., Mulvihill, E. R. & Chambon, P. *Nature* **310**, 680–686 (1983).
- Zimmerman, J. L., Petri, W. & Meselson, M. *Cell* **32**, 1161–1170 (1983).
- Kornberg, R. D. *Nature* **292**, 579–580 (1981).
- Church, G. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* (in the press).

Two types of binary radio pulsars with different evolutionary histories

E. P. J. van den Heuvel

Astronomical Institute, University of Amsterdam, Roetersstraat 15,
1018 WB Amsterdam, The Netherlands

R. E. Taam

Department of Physics and Astronomy, Northwestern University,
Evanston, Illinois 60201, USA

The four known binary radio pulsars (Table 1) seem to fall into two different categories. Two of them, PSR0655+64 and PSR1913+16, have short orbital periods (<25 h)^{1,2} and high mass functions, indicating companion masses $>0.8 M_{\odot}$ (respectively, about $1 M_{\odot}$ and $1.4 M_{\odot}$ (ref. 2)). The other two, PSR0820+02 and PSR1953+29, have long orbital periods (≥ 120 days), nearly circular orbits and low almost identical mass functions of $\sim 3 \times 10^{-3} M_{\odot}$, suggesting companion masses of $\sim 0.2\text{--}0.4 M_{\odot}$ (refs 3–5). We point out here that these two classes of systems are expected to be formed by the later evolution of binaries consisting of a neutron star and a normal companion star, in which the companion was (considerably) more massive than the neutron star, or less massive than the neutron star, respectively. Mass transfer from an evolved companion that is more massive than the neutron star (more precisely, mass ratio ≥ 0.85 (refs 6, 7)) tends to be unstable and to lead to runaway mass transfer and spiral-in resulting in a very short orbital period, whereas mass transfer from an evolved companion that is less massive than the neutron star is stable and leads to expansion of the orbit^{6,7}. Furthermore, we point out that in the systems with a less massive companion, the neutron star most probably was formed by the accretion-induced collapse of a white dwarf. Such a model explains in a natural way why PSR1953+29 has a millisecond rotation period and why PSR0820+02 has not.

When the companion has a mass several times that of the neutron star ($\geq 3\text{--}4 M_{\odot}$, see below) and the system is relatively wide (orbital period $\geq$ a few weeks) so that at the onset of the mass transfer the companion has a deep convective envelope, runaway mass transfer is unavoidable. This is because (1) mass transfer from the more massive to the less massive star leads to shrinkage of the Roche lobe of the more massive star, while (2) a convective envelope tends to expand when the star loses mass⁸. This will, on a time scale of $\sim 10^3$ yr, lead to the formation of an extended convective common envelope in which the neutron star and the evolved core of the companion will spiral towards each other as a consequence of the large frictional drag^{9–12}. The duration of this spiral-in process, in which finally the envelope is lost, is expected to be short, $\sim 10^3\text{--}10^5$ yr (refs 11–13).

The final result is expected to be a system consisting of the neutron star and the evolved core of the companion with an orbital period ranging from ~ 1 h to a few days (in analogy to the case of cataclysmic variables which are products of a similar type of spiral-in evolution^{9,12}).

In order to have deep spiral-in, the two components should differ sufficiently in mass, preferably by more than a factor of two, because otherwise soon after the onset of the mass transfer the mass ratio will be reversed which would lead to subsequent spiral-out. (For systems with mass ratios in the range 0.85–2 the outcome of the evolution is more complex and, for the sake of argument, will not be considered here.)

Figure 1 depicts as an example the anticipated evolution of a binary initially consisting of a $M \approx 5 M_{\odot}$ star and a neutron star, with an orbital period of ≥ 80 days. The $5 M_{\odot}$ star overflows its Roche lobe when it has exhausted helium in its core (that is, when it climbs up along the asymptotic giant branch, AGB).

At helium exhaustion it has a $0.95 M_{\odot}$ degenerate CO core¹⁴ surrounded by He and H-burning shells. During its ascent of the AGB this core mass increases to $1.39 M_{\odot}$. We assume that the star engulfs its companion when $M_{\text{core}} \approx 1.0 M_{\odot}$, implying a binary period of about 100 days (see Table 2). The system after spiral-in will consist of a $1.0 M_{\odot}$ CO white dwarf and a neutron star with a narrow and a circular orbit, that is, closely resembling the PSR0655+64 system (see Table 1). The type of binary evolution considered here is the so-called case C^{6,7}. (In shorter-period systems—case B—the evolution will be somewhat more complex and may lead to several stages of mass transfer^{15,16}; the outcome will, however, in most cases be roughly similar¹⁵.) Table 2 lists the maximum and minimum orbital periods for case C evolution for companion masses in the range $3\text{--}7 M_{\odot}$, derived from Paczynski's¹⁴ evolutionary tracks. The corresponding CO core masses are also listed.

Table 2 shows that an evolution similar to the one depicted in Fig. 1 is expected in all wide (case C) neutron star binaries in which the mass of the companion is in the range ~ 5 to $\sim 8 M_{\odot}$, as on the AGB all such stars develop degenerate CO cores with a mass in the range $0.95\text{--}1.39 M_{\odot}$ (refs 14, 17, 18). For companion masses of $3\text{--}5 M_{\odot}$, the same holds if the binary periods are ≥ 80 days, such that after helium exhaustion the core can still grow to $\geq 0.90 M_{\odot}$ before the star overflows its critical lobe. Companions more massive than $\sim 8\text{--}10 M_{\odot}$ will develop evolved cores too massive to terminate as white dwarfs^{15,19}. Following the spiral-in and subsequent nuclear evolution such a core is expected to collapse to a neutron star. The mass ejection in this second supernova in the system may induce a considerable orbital eccentricity or may even disrupt the system^{20,21}. PSR1913+16 is expected to have been formed in this way^{21,22} (in the case of disruption, two runaway neutron stars will be formed, one young and one old).

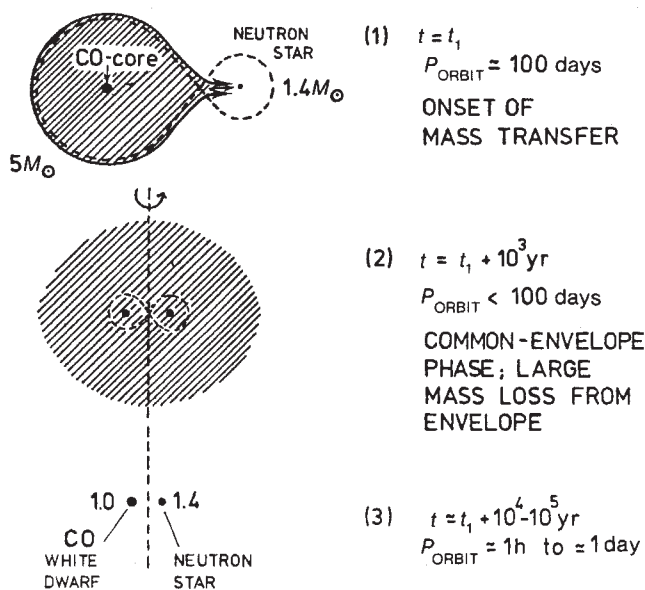


Fig. 1 Anticipated evolution of a relatively wide binary consisting of a $5 M_{\odot}$ star with a neutron star companion. At the onset of the mass transfer the $5 M_{\odot}$ star is a giant with a $1 M_{\odot}$ degenerate CO core and a gradually expanding envelope (1). Rapidly, a common convective envelope forms (2) in which the CO-core and the neutron star spiral-in. During this spiral-in the envelope is ejected due to frictional heating and a very close system remains (3) consisting of a neutron star and a massive CO-white dwarf in a circular orbit. All wide (case C) neutron star binaries with companions in the range $3 M_{\odot}$ to $8\text{--}10 M_{\odot}$ are expected to evolve in this way. For companions more massive than $8\text{--}10 M_{\odot}$ the evolved core after spiral-in collapses to a neutron star, and a close neutron star binary with an eccentric orbit (or two runaway pulsars) will be formed^{20–22}.

At the onset of the spiral-in the neutron star will often be older than 10^7 yr (companion stars of 5–15 $M_\odot$ need some $(1-5) \times 10^7$ yr to leave the main sequence) such that its surface magnetic field strength may have decayed by several orders of magnitude. (The space distribution of radio pulsars with known proper motions indicates that the dipole strength of the surface magnetic field of a neutron star decays on a time scale of $(2-5) \times 10^6$ yr (refs 23–25).) With a canonical magnetic dipole strength at birth of $\sim 10^{12-13}$ G (refs 23–25), the dipole strength at the onset of spiral-in may thus be $\sim 10^{10-11}$ G, as observed in PSR0655+64 and PSR1913+16 (see Table 1). During the spiral-in the accretion onto the neutron star will take place near the Eddington-limited rate $\dot{M}_{\text{Edd}}$, implying that its rotation will be spun-up to a minimum period that depends practically only on the surface dipole strength B_0 (in units of 10^9 G) as^{21,26,27}

$$P = (2.7 \text{ ms}) B_0^{6/7} R_6^{15/7} \left\{ \frac{M}{1.4 M_\odot} \right\}^{-5/7} \left\{ \frac{\dot{M}_{\text{Edd}}}{\dot{M}} \right\}^{3/7} \quad (1)$$

where R_6 is the neutron star radius in units of 10^6 cm and M is its mass. After the loss of the common envelope these neutron stars will become observable as radio pulsars with a relatively low surface dipole magnetic field strength^{21,22,24}. For the observed B_0 values of PSR0655+64 and PSR1913+16 (Table 1), and $R_6 = 1$, $M = 1.4 M_\odot$, equation (1) yields $P = 0.17$ and 0.04 s, respectively, in reasonable agreement with their observed pulse periods (allowing for some spin-down since their formation^{21,24}).

The absence of radio pulses from the second (younger) neutron star in the PSR1913+16 system may be due either to beaming effects, or to the relatively short spin-down time scale of a newborn strong-magnetic field neutron star (if the second supernova in the system took place a few million years ago, the new neutron star may already have reached the turn-off period of a few seconds whereas the 'recycled' old one, due to its weaker surface dipole field, will remain observable as a pulsar for a much longer time^{21,22,27,28}). The best known progenitor candidates for the above described type of evolution seem to be the B-emission X-ray binaries, which have orbital periods ranging from 15 days to several years^{29,30}, and companion masses ranging from $\sim 7 M_\odot$ (spectral type B3Ve) to $\sim 20 M_\odot$ (spectral type O9Ve). The companion of PSR0655+64 should have had a mass below the limit for exploding as a supernova, $\leq 8-10 M_\odot$ (in case C; in case B: $\leq 10-12 M_\odot$) whereas that of PSR1913+16 had a mass above this limit (see ref. 21 for details).

We now turn to the alternative case in which the system started out with a companion star less massive than the neutron star. When in this system (mass ratio ≤ 0.85) the companion begins to overflow its Roche lobe, the ensuing mass transfer will be self-stabilizing as it leads to expansion of the orbit. Webbink *et al.*³¹ and Taam³² have shown that in this way, starting with companions with $M \leq 1.2 M_\odot$ and orbital periods upwards from about 1 day, one obtains a long-lasting ($\sim 10^6-10^8$ yr) stage of relatively high mass transfer ($\dot{M} \geq 10^{-8} M_\odot \text{ yr}^{-1}$).

During this phase the companion ascends the giant branch and has a degenerate helium core with a mass in the range $0.2-0.45 M_\odot$, and generates energy by burning hydrogen in a shell around this core. The mass transfer, and the associated expansion of the orbit, are driven by the gradual growth of the core mass. The duration of the mass transfer phase depends mainly on the initial mass of the companion and the initial

orbital period. In the end, only the degenerate helium core of the companion is left behind, as a helium white dwarf, and the final orbit will always be wide, as the mass and orbital angular momentum of the system are, in first approximation, expected to be conserved.

Savonije³³, Paczynski³⁴ and Joss and Rappaport³⁵ have, independently, pointed out that the final systems resulting from this evolution show a striking resemblance to that of the 6-ms binary radio pulsar PSR1953+29, as (1) the final companion mass will be low ($\sim 0.3 M_\odot$) and (2) the orbit will be (almost) circular as a consequence of the long-lasting preceding mass transfer stage. (When the companion is a red giant which is filling its Roche lobe, tidal dissipation in its convective envelope will be very efficient^{36,37}.) Rappaport and Joss³⁵ showed that for an initial mass of the companion of $1 M_\odot$, the most likely initial orbital period of PSR1953+29 was around 10 days and that of PSR0820+02 about 1 yr. The duration of the mass-transfer phase in these systems was $(3.5-7.7) \times 10^7$ yr and 4×10^6 yr, respectively (similar results are obtained for a companion mass of $1.2 M_\odot$, as can be easily verified).

The above outlined evolutionary model for these wide systems thus seems to explain their observed orbital characteristics, including their mass functions; but this model at the same time implies that the systems must be very old (at least several times 10^9 yr) as the companion stars should have started out with masses $\leq 1.2 M_\odot$ and had already completed their main-sequence evolution before the onset of the mass transfer. Because surface dipole components of magnetic fields of neutron stars seem to decay on a time scale $\leq 10^7$ yr one would, therefore, not expect any detectable surface dipole fields to be left in them.

However, PSR0820+02 and PSR1953+29 still have a surface dipole magnetic field strength of 3×10^{11} G and $> 10^9$ G, respectively (Table 1). The same problem exists in the GX 1+4 system which is an 120 s X-ray pulsar, and must be a very wide system as the companion star is an M6IIIe red giant³⁸. Such a system seems an excellent progenitor candidate for a system like PSR0820+02 (ref. 39). From its spin-up rate in combination with its X-ray luminosity of $\sim 10^{38} \text{ erg s}^{-1}$ (ref. 38) one derives a lower limit to its magnetic field strength of $\sim 3 \times 10^{11}$ G (ref. 26), which is similar to that of PSR0820+02. Because an M6IIIe giant cannot be more massive than $\sim 3 M_\odot$ (ref. 14), the age of this system is at least $\geq 2 \times 10^8$ yr (the main-sequence lifetime of a $3 M_\odot$ star). Moreover, the position close to the galactic centre and at high z suggests an old-disk population old age of $(5-10) \times 10^{10}$ yr.

The only consistent way in which one may explain why the neutron stars in these old (wide) systems still can have such fairly strong surface magnetic fields seems to be if they were formed only recently—during the mass-transfer stage itself, by the accretion-induced collapse of a (relatively massive) white dwarf. (It seems reasonable to assume that, regardless of the formation mechanism, all neutron stars are formed with a canonical surface dipole magnetic field strength of 10^{12-13} G, due to dynamo action during the collapse process²⁵.) Because in the case of PSR1953+29 the mass transfer stage lasted $(3.5-7.7) \times 10^7$ yr and for PSR0820+02 it lasted only $\sim 4 \times 10^6$ yr, the time elapsed since the collapse in these systems may be relatively short: a few times 10^7 yr in the case of PSR1953+29 and $< 10^7$ yr in the case of PSR0820+02, respectively. Hence, one would not expect their surface magnetic fields to have decayed by more

Table 1 Orbital and pulse properties of the four binary radio pulsars together with estimates of their surface magnetic field strengths and the masses of their companions

Name	P_{orb} (day)	e	Mass function ($M_\odot$)	Most likely companion mass ($M_\odot$)	P_{pulse} (s)	B_0 (G)	Ref.
PSR1913+16	0.32	0.617	0.1322	1.40 ± 0.05	0.059	2×10^{10}	1
PSR0655+64	1.03	0.000	0.0712	1.00 ± 0.30	0.196	8.6×10^{10}	2
PSR0820+02	1,232	0.012	0.00301	0.2–0.4	0.865	3.3×10^{11}	4
PSR1953+29	~ 120	< 0.05	0.00272	0.2–0.4	0.006	$\sim 2 \times 10^9$ (predicted)	5, 47

Table 2 Minimum and maximum orbital periods for neutron star binaries in which the companion overflows its critical lobe after it has exhausted helium in its core (case C evolution)

Companion mass	$P_{\min}$ (day)	M_{core} (He exhaustion) ($M_{\odot}$)	$P_{\max}$ (yr)	M_{core} (max) ($M_{\odot}$)
$3M_{\odot}$	32	0.51	5.5 (19)	1.39
$5M_{\odot}$	76	0.95	4.5 (13)	1.39
$7M_{\odot}$	173	1.02	3.6 (10)	1.39

A neutron star mass of $1.40 M_{\odot}$ was assumed and the corresponding masses M_{core} of the degenerate CO-core of the companion are indicated (Paczynski's¹⁴ evolutionary tracks were used). The first value of $P_{\max}$ corresponds to the moment at which the orbital separation equals the radius of the companion; the value within parentheses corresponds to the moment at which the companion fills its Roche lobe.

than a factor of 10^3 and 10^1 , respectively. That PSR0820+02 indeed still has the strongest magnetic field of the two is therefore in excellent agreement with the prediction of this accretion-induced collapse model.

In addition, the conditions for achieving accretion-induced collapse are expected to be favourable just in systems with a low-mass ($\leq 1.2 M_{\odot}$) giant component for the following reasons. To reach collapse the white dwarf (consisting of CO or O–Ne–Mg) should be able to grow substantially by accretion—as the *a priori* probability that it was born with a mass close to the Chandrasekhar limit will be very low. Just at the accretion rates produced by low-mass giant companions (in the range $10^{-8} M_{\odot} \text{ yr}^{-1}$ to $2 \times 10^{-7} M_{\odot} \text{ yr}^{-1}$) a considerable growth of the white dwarf is indeed possible as the accreted hydrogen burns in shell flashes that are too weak to cause mass ejection^{40–42}. At lower accretion rates, $< 10^{-8} M_{\odot} \text{ yr}^{-1}$, hydrogen burns with very strong nova-like flashes in which part (and possibly all) of the accreted matter is ejected such that a substantial growth of the white dwarf is unlikely. In addition, at the age of these systems ($\geq 10^9$ yr) the oxygen in a carbon–oxygen white dwarf may have separated from the carbon and have settled in the core⁴³, creating a situation favourable for the occurrence of accretion-induced collapse⁴⁴.

Because, at any time during the accretion phase the envelope mass of the white dwarf is small (more than a few hundredths of a solar mass of hydrogen would not fit inside its Roche lobe) and because the mass loss of the white dwarf during the collapse need not exceed the equivalent of the binding energy of the neutron star ($\sim 0.1 M_{\odot}$), the orbital eccentricity induced by the mass loss in the collapse need not exceed 0.05 (ref. 20) and the orbit may be subsequently circularized by the continued mass transfer from the companion and by tidal forces^{39,45}.

The above described model is entirely consistent with the very short (6 ms) pulse period of PSR1953+29, because in the PSR1953+29 system the collapse can have occurred several times 10^7 yr before the end of the mass-transfer phase. Consequently, the surface dipole field had time to decay to $\leq 2 \times 10^9$ G and according to equation (1), spin-up to ~ 5 ms was possible. On the other hand, in view of the much shorter mass-transfer time scale in the PSR0820+02 system, such a field decay and spin-up to a short period were not possible here.

Thus we conclude, on the basis of the above arguments, that the existence of the two binary radio pulsars with wide circular orbits and low-mass functions, provides the first very strong evidence that neutron stars can be formed in an old stellar population by the accretion-induced collapse of a white dwarf in a relatively wide binary (Whelan–Iben model⁴⁶).

We thank H. Henrichs, G. J. Savonije, J. van Paradijs, V. Radhakrishnan, R. Blandford and M. Goss for stimulating comments and discussions, V. Boriakoff for providing information on PSR1953+29 in advance of publication, and V. Trimble for suggestions. This work started during the CECAM workshop on "Late Stages of Close Binary Evolution" in Meudon (France), July/August 1982.

Received 28 November 1983; accepted 1 March 1984.

- Taylor, J. H. & Weisberg, J. M. *Astrophys. J.* **253**, 908–920 (1983).
- Damashak, M., Backus, P. R., Taylor, J. H. & Burkhardt, R. *Astrophys. J. Lett.* **253**, L57–L60 (1982).
- Taylor, J. H. *IAU Symp.* **95**, 361–369 (1981).
- Manchester, R. N. et al. *Astrophys. J.* **268**, 832–836 (1983).
- Boriakoff, V., Bucccheri, R. & Fanci, F. *Nature* **304**, 417–419 (1983).
- Plavec, M. *Adv. Astr. Astrophys.* **6**, 201–278 (1968).
- Paczynski, B. A. *Rev. Astr. Astrophys.* **9**, 183–208 (1971).
- Paczynski, B. & Sienkiewicz, R. *Acta Astr.* **22**, 73–91 (1972).
- Paczynski, B. *IAU Symp.* **73**, 75–80 (1976).
- Webbink, R. F. *IAU Colloq.* **53**, 426 (1979).
- Taam, R. E., Bodenheimer, P. & Ostriker, J. P. *Astrophys. J.* **222**, 269–280 (1978).
- Meyer, F. & Meyer-Hofmeister, E. *Astr. Astrophys.* **78**, 167–176 (1979).
- Bodenheimer, P. & Taam, R. E. *Astrophys. J.* (in the press).
- Paczynski, B. *Acta Astr.* **20**, 47–58 (1970).
- Habets, G. *IAU Symp.* **105** (in the press).
- Delgado, A. J. & Thomas, H. C. *Astr. Astrophys.* **96**, 142–145 (1981).
- Iben, I. A. *Rev. Astr. Astrophys.* **12**, 215–256 (1974).
- Becker, S. A. *Astrophys. J. Suppl. Ser.* **45**, 475–505 (1981).
- Van den Heuvel, E. P. J. *IAU Symp.* **93**, 155–175 (1981).
- Flannery, B. P. & Van den Heuvel, E. P. J. *Astr. Astrophys.* **39**, 61–67 (1975).
- Srinivasan, G. & Van den Heuvel, E. P. J. *Astr. Astrophys.* **108**, 143–147 (1982).
- Smarr, L. L. & Blandford, R. *Astrophys. J.* **207**, 574–588 (1976).
- Lyne, A. *IAU Symp.* **95**, 423–436 (1981).
- Radhakrishnan, V. *Proc. Asian Pacific IAU Regional Meet.*, Bandung (ed. Hidayat, B.) (Bandung University, in the press).
- Flowers, E. & Ruderman, M. A. *Astrophys. J.* **215**, 302–310 (1977).
- Henrichs, E. in *Accretion-driven Stellar X-ray Sources* (eds Lewin, W. H. G. & Van den Heuvel, E. P. J.) Ch. 11 (Cambridge University Press, 1983).
- Alpar, M. A., Cheng, A. R., Ruderman, M. A. & Shaham, J. *Nature* **300**, 728–731 (1982).
- Radhakrishnan, V. & Srinivasan, G. *Curr. Sci.* **51**, 1096–1099 (1982).
- Rappaport, S. A. & Van den Heuvel, E. P. J. *IAU Symp.* **98**, 327–346 (1982).
- Priedhorsky, W. C. & Terrell, J. *Nature* **303**, 681–683 (1983).
- Webbink, R. F., Rappaport, A. A. & Savonije, G. J. *Astrophys. J.* **270**, 678–693 (1983).
- Taam, R. E. *Astrophys. J.* **270**, 694–699 (1983).
- Savonije, G. J. *Nature* **304**, 422–423 (1983).
- Paczynski, B. *Nature* **304**, 421–422 (1983).
- Joss, P. C. & Rappaport, S. A. *Nature* **304**, 419–421 (1983).
- Zahn, J. P. *Astr. Astrophys.* **57**, 383–394 (1977); erratum **67**, 162 (1978).
- Savonije, G. J. *Proc. Cambridge NATO Conf. on Interacting Binaries* (Cambridge University Press, in the press).
- Bradt, H. V. D. & McClintock, J. E. A. *Rev. Astr. Astrophys.* **28**, 13–66 (1983).
- Van den Heuvel, E. P. J. in *Pulsars* (eds Sieber, W. & Wielebinski, R.) 371–378 (Reidel, Dordrecht, 1981).
- Sion, E. M., Acierno, M. J. & Tomczyk, S. *Astrophys. J.* **230**, 832–838 (1979).
- Nomoto, K. *Space Sci. Rev.* **27**, 563–570 (1980).
- Nomoto, K. *Astrophys. J.* **253**, 798–810 (1982).
- Stevenson, D. J. *J. Phys. Suppl.* **41**, (3), C2–53 (1980).
- Bravo, E., Isern, J., Labay, J. & Canal, R. *Astr. Astrophys.* **124**, 39–42 (1983).
- Blandford, P. D. & De Campli, W. M. in *Pulsars* (eds Sieber, W. & Wielebinski, R.) 371–378 (Reidel, Dordrecht, 1981).
- Whelan, J. & Iben, I. *Astrophys. J.* **186**, 1007–1014 (1973).
- Helfand, D. J., Ruderman, M. A. & Shaham, J. *Nature* **304**, 423–425 (1983).

Cosmic dust collection by the capture cell technique on the Space Shuttle

J. A. M. McDonnell, W. C. Carey & D. G. Dixon

Space Sciences Laboratory, University of Kent at Canterbury, Kent CT2 7NT, UK

The capture cell technique is a new approach to the study of extraterrestrial material: although complementary to stratospheric collection techniques¹, it avoids selection effects introduced by the deceleration of dust in the Earth's atmosphere. The method, to be used extensively in cosmic dust retrieval experiments on the forthcoming NASA Long Duration Exposure Facility, now yields results from its first application in the microabrasion foil experiment (MFE) which was flown as part of the scientific payload on the STS-3 Space Shuttle mission. Post-flight analysis yielded four hypervelocity impact perforation events during 8 days of exposure to space, and confirmed the capture cell technique as a viable low-cost method for the assessment of the near-Earth particulate environment. The effectiveness of a meteoroid bumper² in dissipating incident particle energy is strikingly demonstrated; markedly different and yet unexplained perforation morphologies are observed and a revision of the current near-Earth microparticle influx model³ is suggested.

MFE was exposed over 8 days in a circular Earth orbit of 241 km altitude and 38° inclination, recording a total of one large (23 μm diameter) and three near-marginal perforations

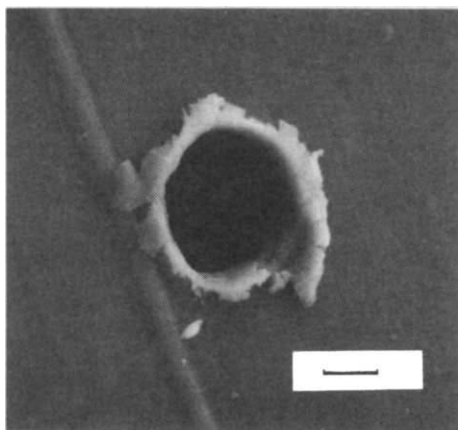


Fig. 1 Scanning electron micrograph of the impact side of the largest aluminium foil perforation (23 μm diameter). Evidence of target melting, raised crater lips and circular symmetry, and especially close correspondence with the exit side (see Fig. 2a) indicates that it was caused by a particle considerably smaller than 23 μm ; at an assumed velocity of 15 km s^{-1} , a particle diameter of 10 μm is required based on simulation and experiment. Scale bar, 10 μm .

(5.1, 6.5 and 7.5 μm in diameter, respectively). The experiment comprises a double layer foil structure⁴; the top layer of 5- μm aluminium foil is held ~ 1 mm above a Kapton sheet by a gold-coated brass spacer grid in a 1- m^2 rectangular array with an effective detection area of 0.4 m^2 . An actual exposure time to space, computed from the shuttle manoeuvring records provides an area-time product of $1.52 \times 10^5 \text{ m}^2 \text{ s}$ (corrected for Earth shielding). Penetration experiments⁵ on identical aluminium foils with iron microparticles show that dust particles with masses $> 10^{-12} \text{ g}$ will marginally penetrate the 5- μm aluminium foil. Particles of lower density would have a larger mass threshold, scaled, for example⁶, according to a dependence on (projectile density)^{1/2}. For marginal perforations, expansion velocities behind the foil are reduced to some small fraction of the initial impact speed, but for those typical of the larger perforation, the particle totally vaporizes⁷ and expands into the capture cell with little reduction of velocity. Impact debris and vapour strike the Kapton sheet over an area much greater than the area of the first perforation. Both particulate matter and vapour from the impact will adhere to the Kapton, but lighter volatiles may condense on both inner surfaces of the capture cell.

Impact perforations in the returned foils were located in the laboratory by a visual inspection system on a light table: pre-

flight records of defects in the foils were compared with identical post-flight inspection and locations of candidates for possible hypervelocity perforations then fully examined in a scanning electron microscope. Tears and defect-associated perforations were rejected. A sensitivity of 3 μm diameter perforation is superior to the lower limit of perforation from hypervelocity impact⁸, thus affording confidence that only four perforations were accumulated.

Figure 1 shows the 'entrance' side of the largest perforation and Fig. 2 the rear (exit) side of all four microparticle perforations. The exit sides show most striking morphological characteristics and also differences between the four events. The entrance morphology of all perforations is very similar, having circular lips extending away from the foil surface as seen in laboratory simulations⁸.

The first and largest perforation detected (Figs 1, 2a) possesses very distinctive hypervelocity impact features, as would be expected from a comparatively large cosmic dust particle. Lips around the hole on both the front and rear foil surfaces are almost identical and demonstrate clear evidence of expansion after the passage of the particle, a feature shown in computer simulations⁷ of impact at 68 km s^{-1} . Radial symmetry is observed and absence of deformation away from the crater lips gives evidence of a particle considerably smaller than 23 μm diameter. On the rear side of the aluminium foil, near the crater, an area of debris is seen, approximately circular with a diameter of 100 μm and centred on the perforation. It consists of sub-micrometre globular particles and micrometre-sized irregular iron-rich particles and may arise from the condensation of impact vapour. It could also contain 'splash-back' from the Kapton as well as condensed foil material. With this perforation, an extensive impact debris zone was found on the underlying Kapton sheet (Fig. 3), of diameter 200 μm but penetrating only to a depth of 1 μm —a marked demonstration of a meteor bumper dissipating the energy of an incident particle and of a high impact velocity. This was offset by some 500 μm from the top crater; from the concentric distribution relative to the perforation rather than the debris, we conclude that it is possible for a fraction of the expanding projectile and target to condense on the rear side of the first foil, but certainly most material impacts the Kapton first.

Returning briefly to the morphology of the three marginal perforations (Fig. 2b, d), distinctive petalling of the perforation lips and often a separation into layers is observed. This is not seen in laboratory simulations (performed with a 2-MV van der Graaff dust accelerator) at this scale. The origin of the feature seen in Fig. 2c, thought perhaps to be an exploded liner of a compressed micrometeoroid, is also unknown, but from energy-dispersive X-ray spectroscopy (EDS) analyses no material other

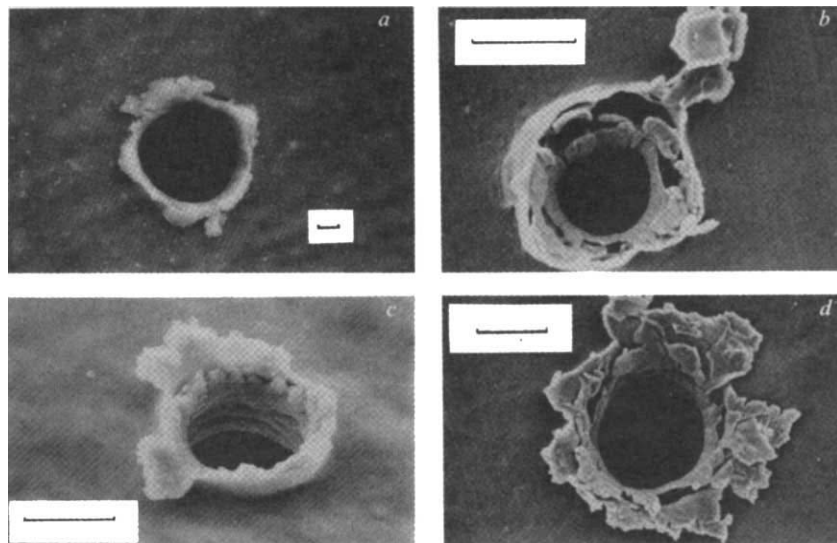


Fig. 2 Exit side of the four perforations showing: a, the largest perforation (23 μm); b–d, three near-marginal perforations of diameter 5.1, 6.5 and 7.5 μm , respectively. Although strikingly different in morphology and unmatched in microscale impact studies, b–d retain evidence of a melted surface of the primary crater 'liner' which is peeled open at the terminal stage of perforation. c could show evidence of particle retention comprising a liner of some 1 μm thickness. Scale bars, 5 μm .

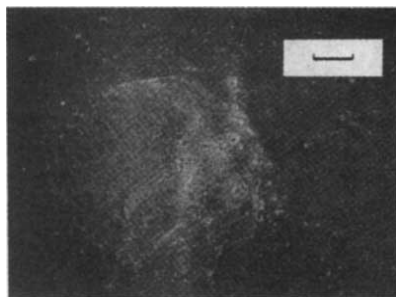


Fig. 3 Second surface debris from the largest crater collected on the Kapton substrate. Damage extends to an area of $200\ \mu\text{m}$ in diameter, but is restricted to $1\ \mu\text{m}$ in depth. Trails of droplets (identified as aluminium from the target) are seen forming both radial streamers and in a hollow conical distribution near the edges of the debris. Scale bar, $50\ \mu\text{m}$.

than aluminium has been detected; it may represent a mixed projectile and target with sufficiently different thermal properties to differentiate on cooling. Initial EDS examinations have, however, revealed silicon surrounding these structures. Full results of these analyses is continuing and will be reported elsewhere, as will a more detailed morphological discussion of the mechanics of penetration. Examining briefly the flux rate measured during the mission, however, we find the data offers significant insight into what could be a different understanding of some near-Earth microparticle flux data.

Figure 4 transposes the penetration data to a mass scale based on penetration experiments⁵ and significant data from deep space and lunar measurements at 1 AU heliocentric distance. Two factors concerning exposure geometries must be clarified before the fluxes may be validly compared, namely sensor angles and flux isotropies. The MFE experiment offers unshielded 2π sr geometry and may be compared with data such as those of the lunar surface without geometric corrections. This configuration has generally been referred to as an area-time product in m^2s 2π sr, and yet considering an element of solid angle $d\Omega$ of a sensor $A\ \text{m}^2$ exposed at an angle θ of a unit sphere, we note that the product of the effective area (A_{eff}) and solid angle ($d\Omega$) is $A_{\text{eff}}d\Omega = A \cos \theta\ 2\pi \sin \theta\ d\theta$. Hence the total area-time product is

$$\int_0^{\pi/2} A_{\text{eff}} d\Omega = 2\pi A \int_0^{\pi/2} \cos \theta \sin \theta\ d\theta = \pi A\ \text{m}^2\text{sr}$$

We should thus relate an unshielded flat sensor to a true exposure solid angle of π sr. We therefore now scale the directional measurements from sensors of solid angle Ω_p by the ratio π/Ω_p and note that the lunar data need not be changed numerically, but do refer to π sr exposure. The Pioneer 8 and 9 deep space interplanetary data were previously incorrectly scaled³ by the ratio $2\pi/\Omega$. Such corrections have the effect of reducing measurements of the interplanetary flux by a factor of 2 (Fig. 4). For anisotropic fluxes, we note that at masses of $\sim 10^{-11}$ g or greater, the data show³ a strong predominance towards an Earth apex flux from the forward orbital motion of the near-circular orbit of the Earth into slower particles near their aphelion⁹. To such a flux, a rotating sensor of area A offers an effective area of

$$\frac{2}{\pi} \int_0^{\pi/2} A \cos \theta\ d\theta = \frac{2A}{\pi}$$

over angles $\pm \pi/2$ radians to the apex direction and 0 for $\pm \pi/2$ to $\pm \pi$ radians (shielded), yielding an average of $A/\pi\text{m}^2$. Hence, to estimate the average flux intercepted by sensor rotating in an apex dominant 'stream' we must multiply such measured apex flux values by $1/\pi$. Gravitational enhancement¹⁰ in comparing lunar and near-Earth data must be considered, but this is not included in Fig. 4.

The relationship between foil thickness penetrated and particle mass/crater pit diameter is found⁵ by assuming a particle

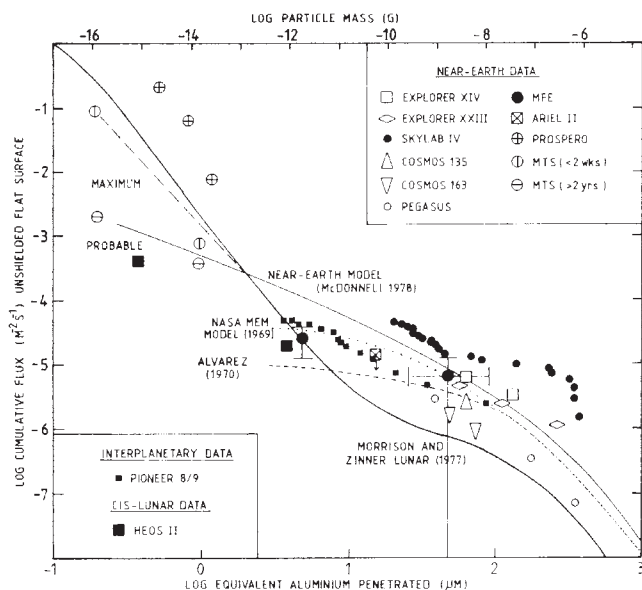


Fig. 4 Relevant flux measurements in the vicinity of 1 AU heliocentric distance³ corrected for Earth shadowing and reduced to a flat surface exposure geometry. The MFE yields a flux of $\Phi = 2.6 \times 10^{-5}\ \text{m}^{-2}\text{s}^{-1}$ (flat surface sensor of π sr effective exposure angle corrected for Earth shielding) for a perforation thickness of $5\ \mu\text{m}$ aluminium, or $m = 1.8 \times 10^{-12}$ g particle mass; it differs significantly from previous models and supports a more pronounced flattening of the size distribution at masses 10^{-12} g. If the lunar data and the near-Earth Prospero measurements are reliable indicators of the incident flux in Earth orbit, the trend would (shown above on a cumulative plot) support the existence of a bimodal particle distribution¹²; the significance of secondary cratering in lunar data at low masses must be addressed.

density of $3\ \text{g cm}^{-3}$ and a velocity of $20\ \text{km s}^{-1}$, which results in a $1\text{-}\mu\text{m}$ crater pit diameter being equivalent to $2.33\ \mu\text{m}$ of aluminium. The MFE data in Fig. 4 are plotted at a measured cumulative penetration flux for $t = 5\ \mu\text{m}$ foil thickness for the four perforations; also estimated is the foil thickness which the particle forming the largest perforation would have perforated, namely $t = 48\ \mu\text{m}$. This perforation is estimated by impact data⁵ to have been caused by a particle of diameter $10\ \mu\text{m}$ at an assumed velocity of $15\ \text{km s}^{-1}$. A recent near-Earth flux model³, which interpolated through the region of sparse penetration data, is now seen to have been overestimated at masses in the region of 10^{-12} g, probably by one magnitude and at least by a factor of five. Despite the small number of hypervelocity events recorded by MFE, we stress that penetration is a highly reliable sensing technique. An exaggeration of the flux value may occur if, for example, foil defects are falsely attributed to extraterrestrial impacts, but the crater morphology precludes this possibility; scanning by two observers has established the efficacy of the MFE detection with regard to the possibility of missing perforations. Even the statistical uncertainties of the flux measure based on four events, yielding an accuracy of $\pm 50\%$, are insufficient to avoid clear indications that a lower near-Earth penetration environment should be considered.

In a wider context, the less sensitive Explorer and Pegasus data compare well with lunar data⁹, showing a near-Earth enhancement of the flux by less than an order of magnitude. At small masses, however, the interpretation of the MFE data in conjunction with data from Heos II¹¹ presents some difficulty, as the *in situ* measured flux is clearly lower than the lunar data of Morrison and Zinner⁹. Le Sergeant and Lamy¹², in considering the 'knee' of the lunar distribution, suggested that the derived primary microcrater distribution of Flavill *et al.*¹³ supported the presence of two different interplanetary dust populations as first proposed by Brownlee *et al.*¹⁴. This work¹³ decoded the obser-

ved microcrater distribution on lunar sample 12054 to remove the contribution from secondary craters, resulting in a flatter slope for the distribution in the region of 10^{-12} g. In a similar manner, an attempt has been made¹⁵ to deconvolve the lunar data at lower masses leading to a reduction in the observed flux of a factor of 2 at a mass of 10^{-13} g and an order of magnitude at a mass of 10^{-16} g.

The need for such possible corrections to the microparticle influx derived from observed lunar microcrater distributions at masses $<10^{-12}$ g is now firmly supported by the MFE data. The experiment also exposes difficulty in understanding how the Prospero flux at mass 2×10^{-14} g is 2.5 magnitudes higher than the MFE data at mass 10^{-12} g. A logarithmic cumulative mass slope of ~ 1.5 is called for which is higher than any measured interplanetary or lunar microparticle flux data distributions. Although changing particle density could affect the comparison of penetration and such impact plasma experiments, the agreement between MFE and the Heos II plasma derived data argues against this, as does evidence from lunar microcraters where

extremes of particle density or shape factor are shown to be atypical¹⁶.

Impact craters recovered from Skylab windows¹⁷ which showed evidence of aluminium microspheres in Earth orbit probably derived from upper-stage rocket firings, shows (Fig. 4) a flux distribution of anomalous slope and magnitude. The MFE data argue against this population being dominant at micrometre dimensions at least at the time of the STS-3 flight. The attribution of the four impacts to natural interplanetary sources, which is supported by chemical analyses, already yields a lower flux than previous data; we must conclude that at these dimensions the capture cell technique is likely to return a pre-dominance of extraterrestrial material.

Acknowledgements and thanks are due to the NASA OSS-1 team and Ken Kissin, to J. L. Weinberg for development of the MFE flight opportunity, and to SERC for financial support. We also thank the applications laboratories of JEOL(UK), Cambridge Instruments, Philips Electron Optics and Hitachi Electron Optics for assistance and advice.

Received 14 November 1983; accepted 16 February 1984.

- Bradley, J. P., Brownlee, D. E. & Veblen, D. R. *Nature* **301**, 473–477 (1983).
- Whipple, F. L. in *Physics and Medicine of the Upper Atmosphere*, 137–170 (University of New Mexico Press, Albuquerque, 1952).
- McDonnell, J. A. M. in *Cosmic Dust*, Ch. 6 (Wiley, New York, 1978).
- McDonnell, J. A. M., Carey, W. C. & Dixon, D. G. *Lunar planet Sci.* **14**, 475–476 (1983).
- McDonnell, J. A. M. in *The Comet Halley Micrometeoroid Hazard*, 93–97 (ESA SP-153, Noordwijk, 1979).
- Fish, R. H. & Summers, J. L. *Proc. 7th. Hypervelocity Impact Symp.* VI, 1–26 (Martin, Orlando, 1965).
- Arnaudau, F., Chau, T. & de Rouvray, A. *ESA/ESTEC Report*, Contract 4637/81/NL/AB (1981).

- Grün, E. & Rauser, P. *Space Res.* **9**, 147 (1969).
- Morrison, D. A. & Zinner, E. *Proc. 8th lunar Sci. Conf.*, 841–863 (1977).
- Zook, H. A. *Proc. 6th lunar Sci. Conf.*, 1653–1672 (1975).
- Hoffman, H., Fehlig, H., Grün, E. & Kissel, J. *Planet. Space Sci.* **23**, 215 (1975).
- Le Sergeant d'Hendecourt, L. B. & Lamy, Ph. L. *Icarus* **43**, 350–372 (1980).
- Flavill, R. P., Allison, R. J. & McDonnell, J. A. M. *Proc. 9th lunar Sci. Conf.*, 2539–2556 (1978).
- Brownlee, D. E., Hörz, F., Hartung, J. B. & Gault, D. E. *Proc. 6th lunar Sci. Conf.*, 3409–3416 (1975).
- McDonnell, J. A. M. & Allison, R. J. *Lunar planet. Sci.* **12**, 682–684 (1981).
- Fehlig, H., Grün, E. & Kissel, J. in *Cosmic Dust*, Ch. 9 (Wiley, New York, 1978).
- Clanton, U. S., Zook, H. A. & Schultz, R. A. *Proc. 11th lunar Sci. Conf.*, 2261–2273 (1980).

Proposed structures for poorly characterized phases in C2M carbonaceous chondrite meteorites

Ian D. R. Mackinnon* & Michael E. Zolensky

* Microbeam Inc., Mail Code SN4, NASA Johnson Space Center, Houston, Texas 77058, USA

Recent investigations of meteorite matrices using analytical electron microscopy and high-resolution transmission electron microscopy (HRTEM) have provided data on the structure and chemistry of poorly characterized phases (PCP)¹. We suggest here that ~ 10 Å PCP, a dominant matrix variety, has a structure equivalent to iron-rich tochilinite $[6\text{Fe}_{0.9}\text{S}_5(\text{Fe}, \text{Mg})(\text{OH})_2]$ which consists of coherently interstratified mackinawite and brucite sheets². In addition, we propose that ~ 17 Å PCP, previously described as an SBB-type mixed-layer structure³, is a commensurate intergrowth of serpentine and tochilinite layers. A wide range of cation substitutions is possible within both tochilinite and serpentine-tochilinite structural types. We suggest that various forms of PCP observed in carbonaceous chondrites^{4,5} are intergrowths of tochilinite, serpentine, serpentine-tochilinite and/or vallerite-type minerals⁶.

Early HRTEM studies of the Murchison C2M meteorite^{7,8} revealed that the fine-grained matrix ($<1,000$ Å) has a complex mineralogy containing unusual mixed-layer structures, including an ordered variety termed an SBB-type structure (The term SBB-type structure indicates an ordered arrangement of serpentine-type and brucite-type layers as originally suggested by Mackinnon and Buseck³. Tomeoka and Buseck⁹ denote this phase '7-5-5'; the numbers refer to the lattice fringe spacings in a HRTEM image.) Subsequent work confirmed the presence of SBB-type phases in the Murray, Nogoya and Yamato-74662 meteorites^{10,11} and indicated that Fe may be abundant in this type of phase. Fine-scale chemical analyses (<500 Å) showed that Ni, Al, Si and S are also present¹². Mackinnon¹² suggested that SBB-type structures in the Mighei matrix may be a variety of PCP or PCP intergrown with matrix phyllosilicates. Subsequently, detailed HRTEM studies by Tomeoka and Buseck^{9,13}

have shown that Mighei matrix PCP includes (1) coherent intergrowths of S-type (~ 7 Å) and B-type (~ 5 Å) layers as an SBB-type structure, (2) disordered ~ 5 Å and ~ 7 Å layers and (3) an ordered ~ 5 Å phase. Recent lattice image studies of C2M matrices^{13,14} have emphasized an ordered ~ 5 Å phase in their analyses of PCP, although there is some ambiguity in the terminology used.

A compilation of data (colour, optical anisotropy, reflectivity, hardness and morphology) for typical PCPs from C2M meteorites^{4,5,15} and for terrestrial tochilinite¹⁶ shows that in all cases, properties of fibrous PCP match the bulk properties of fibrous tochilinite. A similar case can be made for the many varieties of PCP and tochilinite. Electron microprobe analyses⁵ have shown that PCP morphologies are correlated with composition: a massive, sulphur-rich Fe, Ni phase; a granular to formless Mg, Si-rich Fe sulphide and a fibrous Fe, Ni phase with intermediate amounts of Si, S and C. Tochilinite is usually fine-grained in terrestrial occurrences and is difficult to analyse accurately; representative microprobe analyses from two localities are given in Table 1. For comparison, columns 3 and 4 of Table 1 also show microprobe analyses for typical PCP phases in C2M chondrites. In general, major element chemistry for tochilinite agrees well with analyses of PCP from C2M meteorite matrices.

In their study of the ~ 10 Å PCP phase, Barber *et al.*¹ present a set of interplanar spacings from a (presumed) ordered single crystal. These spacings, obtained from conventional electron diffraction and optical transforms of structure images, are not as precise as X-ray powder diffraction data. Nevertheless, a compilation of interplanar spacings for tochilinite¹⁶ and the ~ 10 Å PCP described by Barber *et al.*¹ show remarkable agreement (Table 2). One exception in this compilation is the 7.7 Å (and perhaps 2.9 Å) spacing(s), which may be due to a small amount of serpentine-type mineral interstratified with the ordered ~ 10 Å PCP phase. For comparison, Table 2 also gives interplanar spacings for lizardite¹⁷ and vallerite¹⁸. X-ray patterns from a fibrous variety of PCP in Murchison also give a sharp line at 5.4 Å and a weaker line at 2.72 Å (ref. 4). Barber *et al.*¹ suggest a chemical formula of $\text{Fe}_{1.4}\text{SO}_{1.3}$ for ~ 10 Å PCP using electron energy loss spectroscopy analysis. This method is not as precise as conventional electron microprobe analysis¹,

Table 1 Reported microprobe analyses of terrestrial tochilinites and meteoritic PCPs

Wt%	Tochilinite ¹⁶		Meteoritic ⁵	
	1	2	3	4
Fe	44.92	35.27	40.03	41.97
Mg	10.94	13.24	0.42	0.34
Al	—	4.27	0.28	0.05
S	21.73	24.33	17.00	20.40
Cr	—	—	2.26	—
Ni	—	—	7.70	5.8
C	—	—	2.2	2.4
Other	—	—	1.33	0.45
Total	77.59	77.11	71.22	71.41

'Other' includes minor amounts of Ca, K, P and Si. Columns 3 and 4 are PCP type I and PCP from a CM clast in the Jodzie meteorite, respectively⁵. Analyses from ref. 5 are converted to wt% elements to facilitate comparison with tochilinite values. All columns do not add up to 100% due to the presence of structural water which has not been analysed. For example, in column 1, a calculated (OH) content of 20.99 wt% yields a total analysis of 98.58%¹⁶.

but within the relative error of the technique (~16%) and expected chemical variations (see below), there is agreement with the accepted tochilinite formula. Indeed, based on this analysis, Barber *et al.*¹ suggest that ~10 Å PCP may be the fibrous mineral described by Jambor¹⁶, subsequently identified as tochilinite².

Selected area electron diffraction studies² show the complexity of valleriite- and tochilinite-like minerals, which consist of alternating sulphide and brucite sheets. Figure 1a shows an idealized model of the tochilinite structure along the *c* axis. In this structure, brucite-type sheets alternate with mackinawite-type sulphide sheets to form a C-centred unit cell with dimensions *a* = 5.37 Å, *b* = 15.60 Å, *c* = 10.72 Å and $\beta = 95^\circ$ (ref. 2). The mackinawite sheet consists of FeS₄ tetrahedra in a unique edge-shared arrangement. The relative rotation of neighbouring brucite sheets is 22°, while each brucite sheet is also rotated relative to the origin of the mackinawite sheet².

Individual sheets within the tochilinite structure can be considered as being structural units bound together primarily by hydrogen bonds. Consequently, cation substitutions common to brucite and mackinawite can be expected for the compositional range of tochilinite. Chemical formulae on the right-hand side of Fig. 1a show the major cation substitutions expected within each sheet of the tochilinite structure. The mackinawite sheet can accommodate a high concentration of vacancies as well as substitutions of other cations¹⁹. Analyses of Kamaishi mine samples¹⁹ show that the tochilinite structure allows significant Cu substitution within individual mackinawite sheets. However, higher contents of Cu within the sulphide sheet stabilize the valleriite structure. In valleriite, the sulphide sheet is a derivative of the cubic close-packed chalcopyrite structure containing edge-shared Cu, Fe tetrahedra²⁰. Analyses of other tochilinites from Kamaishi indicate that considerable amounts of Fe and minor amounts of Al and Ca may substitute into the brucite sheet¹⁹. Indeed, the replacement of Fe for Mg in the brucite structure is a well-documented phenomenon and may range from 6 to 72 mol% Fe(OH)₂ (ref. 21 and refs therein); the latter is known as amakinite²². Within tochilinite, vacancies in the sulphide sheet produce a net negative charge, but this can be balanced by the presence of trivalent Fe in the brucite sheet^{2,19}. Addition of minor amounts of Ni within the sulphide sheet may allow unusual charge balance distributions which may depend on the environment of formation. On the basis of carbonaceous chondrite chemical analyses^{4,5}, we suggest that meteoritic tochilinite will tend to form iron-rich tochilinite structures with alternating mackinawite and amakinite sheets.

HRTEM observations show that ~10 Å PCP forms sinuous, curved, rolled and 'corrugated' structures in the Mighei matrix^{1,13,14}. These observations of layer curvature can be explained by the degree of misfit between two sheets of different

Table 2 Reported interplanar spacings (Å) for meteoritic PCP and terrestrial layer structure minerals

Meteoritic PCP* (ref. 1)	Tochilinite † (ref. 16)	Valleriite† (ref. 18)	Lizardite† (ref. 17)
10.8	10.84	11.4	
7.7			7.4
5.4	5.41	5.71	
	4.13		4.6
3.6	3.61	3.80	3.9
3.5	3.46	3.27	3.67
	3.15	3.23	
2.9	2.96	3.07	2.875
2.7	2.625	2.846	2.663
	2.562	2.604	2.505
2.4	2.336	2.346	2.410
	2.307		2.307
2.25	2.227	2.259	2.156
2.1	2.103		
	2.072	2.041	
	2.051	1.885	1.945
	1.861	1.860	1.835
	1.814	1.780	1.799
	1.751		1.743
1.65	1.616	1.629	1.692

* Electron diffraction data.

† X-ray diffraction data.

sizes. Within individual sheets, cation substitution will change the degree of misfit, and thus the radius of sheet curvature will also vary. The greatest degree of misfit is along the *a* axis of tochilinite². Therefore, varieties of tochilinite curved about the *a* axis will be most common. In addition, the presence of edge dislocations in a plane parallel to the *a* axis of brucite provides a mechanism for the lattice to curve about *a* (ref. 23). Thus, buckling of the tochilinite structure normal to *c*, but not parallel to *a*, will produce a corrugated structure similar to antigorite²⁴. However, unlike antigorite, the mechanism of misfit need not be periodic within the brucite sheet of tochilinite. Thus, corrugations within tochilinite should be limited. Buckling of tochilinite, in concert with cation substitution, may produce a three-dimensional incommensurate structure with layers loosely held

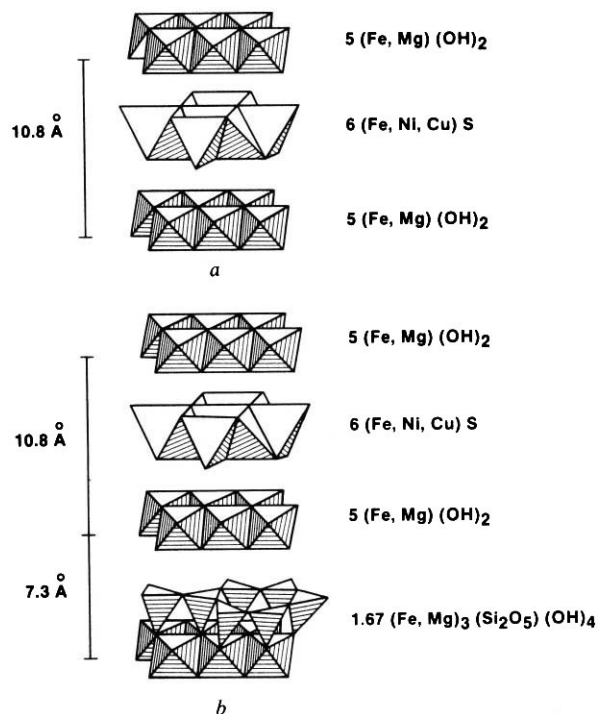


Fig. 1 Schematic models of proposed structures for two predominant types of PCP in C2M meteorites: *a*, a tochilinite structure; *b*, a serpentine-tochilinite structure.

together by hydrogen bonding. Complex structural adjustments of this nature will result in equally complex HRTEM images of meteoritic tochilinite as shown by Barber *et al.*¹ and Tomeoka and Buseck^{13,14}.

We further suggest that the tochilinite structure is a subunit of the SBB-type (~17 Å PCP) phase⁹. HRTEM images of ~17 Å PCP show a distinctive alternation of ~7 Å and ~5 Å lattice fringes of the type SBB (refs 4, 25) or 7-5-5 (ref. 11). These HRTEM images were initially interpreted as ordered alternations of serpentine-type (S) and brucite-type (B) layers on the basis of structural arguments and matrix bulk chemistry^{3,25}. A Si-rich component of some PCPs is also inferred from their relatively high Si contents^{5,12}. A combination of serpentine and tochilinite layers (held together by hydrogen bonds) gives a basal periodicity between ~17 and ~18 Å, depending on the strength of interlayer bonds and/or chemistry. A schematic model of this proposed serpentine-tochilinite structure is shown in Fig. 1b. In this particular case, the planar lizardite layer conforms with the observed planar layer structure of an SBB-type PCP shown in Figs 4 and 5 of ref. 25. Compositional variations within the serpentine layer may also induce a corresponding modulation of the overall structure²⁵. Thus, serpentine-tochilinite structures with a higher Mg content in the 7 Å layer tend to form curved or cylindrical structures as shown in Fig. 1 of ref. 8. However, structural misfit between the mackinawite and brucite sheets of tochilinite can also produce curved, sinuous or 'turbostratic' structures. Therefore, we suggest that variations in *c* axis structure images of serpentine-tochilinite are due to the misfit of octahedral and tetrahedral sheets within both tochilinite and serpentine layers and may also correspond to subtle variations in composition along the layers.

An indication that serpentine-tochilinite may occur in terrestrial rocks comes from studies of a calcite healed breccia in limestone at the Grace Mine, Pennsylvania¹⁶. In these samples, some regions containing tochilinite show a distinctive compositional zoning in which optically darker bands are rich in silica and relatively low in sulphur. Jambor¹⁶ suggests that these zones consist of intimately interlayered tochilinite and serpentine. Microprobe analyses from these regions are similar to analyses of Si-rich PCP in C2M meteorites⁵. We suggest that a detailed HRTEM investigation of Grace Mine samples will provide evidence for a terrestrial occurrence of serpentine-tochilinite. Tochilinite has also been found with a valeriite-like carbonate phase⁶. The presence of carbon in PCPs was suggested by Ramdohr¹⁵ and concentrations as high as 8 wt % have been analysed in PCPs⁷. On the basis of these analyses and the proposed structures of two types of PCP, we suggest that valeriite-type layer structures may also occur in C2M matrices.

Conclusions regarding the petrogenesis of meteoritic tochilinites and related structure types must be developed with caution. Significant compositional differences are apparent between terrestrial and meteoritic tochilinites. For example, Ni is absent from many terrestrial occurrences of tochilinite²², although we consider it possible that minor amounts of Ni can be substituted into the mackinawite sheet. (An indication that Ni-rich tochilinite occurs in terrestrial rocks has recently been given by van der Vusse and Powell²⁶.) The presence of Ni in tochilinites may prove to be an indicator of the environment of formation. In suitable conditions, substitution of other elements (for example, K, P) may also be possible in interlayer sites. Terrestrial tochilinite has been largely ignored by petrologists partly because of its small grain size and (presumed) limited occurrence. However, PCPs (and therefore, meteoritic tochilinites) constitute a significant part of an important group of meteorites, and we urge a strong commitment towards an understanding of phase relations and probable formation mechanisms for tochilinite and serpentine-tochilinite. Furthermore, experimental data obtained in conditions pertinent to carbonaceous meteorite formation should provide significant insight into the origin and evolution of C2M meteorite matrices.

Discussions with J. Jambor and comments on a draft manuscript by F. J. M. Rietmeijer and D. S. McKay are greatly

appreciated. Constructive reviews were given by D. J. Barber and H. Y. McSween. M.E.Z. is a NRC Research Associate at JSC.

Received 25 November 1983; accepted 28 February 1984.

1. Barber, D. J., Bourdillon, A. & Freeman, L. A. *Nature* **305**, 295–297 (1983).
2. Organova, N. I., Drits, V. A. & Dmitrik, A. L. *Am. Miner.* **59**, 190–200 (1974).
3. Mackinnon, I. D. R. & Buseck, P. R. *Nature* **280**, 219–220 (1979).
4. Fuchs, L. H., Olsen, E. & Jensen, K. J. *Smithson. Contr. Earth Sci.* **10**, (1973).
5. Bunch, T. E. & Chang, S. *Geochim. cosmochim. Acta* **44**, 1543–1577 (1980).
6. Harris, D. C. & Vaughan, D. J. *Am. Miner.* **57**, 1037–1052 (1972).
7. Mackinnon, I. D. R. & Buseck, P. R. *Proc. 10th Lunar Sci. Conf.*, 937–949 (1979).
8. Mackinnon, I. D. R. *Proc. 11th Lunar Sci. Conf.*, 839–852 (1980).
9. Tomeoka, K. & Buseck, P. R. *Meteoritics* **17**, 289–290 (1982).
10. McKee, T. R. & Moore, C. B., *Proc. 10th Lunar Sci. Conf.*, 921–935 (1979).
11. Akai, J. in *Proc. 5th Symp. Antarctic Meteorites*, 299–300 (1980).
12. Mackinnon, I. D. R. *Meteoritics* **15**, 328–329 (1980).
13. Tomeoka, K. & Buseck, P. R. *Nature* **306**, 354–356 (1983).
14. Tomeoka, K. & Buseck, P. R. *Proc. Electron Microscopy Soc. Am.*, 188–189 (San Francisco Press, 1983).
15. Ramdohr, P. *The Opaque Minerals in Stony Meteorites* (Academic, New York, 1973).
16. Jambor, J. L. *Geol. Surv. Can.* **76-1B**, 65–69 (1976).
17. Rucklidge, J. C. & Zussman, J. *Acta crystallogr.* **19**, 381–389 (1965).
18. Evans, H. T. Jr *et al.* *U.S. Geol. Surv. Prof. Pap.* **475**, D64–D69 (1964).
19. Muramatsu, Y. & Nambu, M. *J. Jap. Ass. Miner. Petrol. econ. Geol.* **75**, 377–384 (1980).
20. Evans, H. T. Jr & Allmann, R. Z. *Kristallogr.* **127**, 73–93 (1968).
21. Moody, J. B. *Lithos*, 125–138 (1976).
22. Kozlov, I. T. & Levshov, P. P. *Zapiski Vses. Mineral. Obshch.* **91**, 72–77 (1962) in Russian; referenced in *Am. Miner.* **47**, 1218 (1962).
23. Brindley, G. W. & Ogilvie, G. J. *Acta crystallogr.* **5**, 412–413 (1952).
24. Yada, K. *Can. Miner.* **17**, 679–692 (1979).
25. Mackinnon, I. D. R. *Geochim. cosmochim. Acta* **46**, 479–489 (1982).
26. van der Vusse, R. & Powell, R. *Miner. mag.* **47**, 501–505 (1983).

Does the photochemistry of the troposphere admit more than one steady state?

Warren H. White & David Dietz

Center for Air Pollution Impact and Trend Analysis,
Washington University, St Louis, Missouri 63130, USA

Nitrogen oxides are released to the troposphere primarily as NO and NO₂, relatively insoluble gases which are not effectively scavenged by clouds and precipitation¹. Nitrogen oxides are removed from the troposphere primarily as HNO₃, the highly soluble product of reactions with O₃ and various free radical species². The NO_x (=NO+NO₂) removal rate thus depends on the oxidizing potential of the troposphere, which in turn is sensitive to the NO_x concentration³. Here we demonstrate that in certain conditions the NO_x removal rate is a non-monotonic function of NO_x concentration. A consequence is that NO_x emission rates within a critical range can support two or three different steady-state NO_x concentrations. The 'nonstandard' steady states are characterized by low concentrations of ozone and radicals, and by high accumulations of the trace gases these normally scavenge. The emission rates required to support such alternative steady states appear to be somewhat in excess of present levels.

The possibility of multiple steady states is illustrated here in a zero-dimensional (box) model, transport being neglected to emphasize the essentially chemical nature of the phenomenon. Conditions in the box are chosen to be representative of the free troposphere⁴: 273 K, 1.7×10^{19} molecules cm⁻³ (4 km altitude), and 3,000 p.p.m. water vapour.

Table 1 presents the gas-phase reaction scheme, condensed where possible by the elimination of intermediates with unambiguous fates. The rate constants are adapted from Hampson and Garvin⁵ and Chameides⁶; photolysis rates represent equinoctial diurnal averages at 45°N. The effects of heterogeneous reactions are lumped into the first-order rates for physical removal, which are presented in Table 2 together with external source strengths. The degree of nonlinearity in the dependence of NO_x removal on NO_x concentration, and hence the potential for multiple steady states, is controlled by the relative importance of the (nonlinear) chemical and (linear)

Table 1 Reaction scheme used in calculations

	Reaction	Rate coefficient
R1	$\text{NO} + \text{O}_3 \rightarrow \text{NO}_2$	1.5 (+1)
R2	$\text{NO}_2 + h\nu \rightarrow \text{NO} + \text{O}_3$	1.6 (-1)
R3	$\text{NO}_2 + \text{O}_3 \rightarrow \text{NO}_3$	2.2 (-2)
R4	$\text{NO}_3 + \text{NO} \rightarrow 2\text{NO}_2$	2.8 (+4)
R5	$\text{NO}_3 + h\nu \rightarrow \text{NO}_2 + \text{O}_3$	1.4 (-1)
R6	$\text{NO}_3 + h\nu \rightarrow \text{NO}$	1.4 (-1)
R7	$\text{O}_3 + h\nu \rightarrow 2\text{HO}$	9.4 (-6)
R8	$\text{CO} + \text{HO} \rightarrow \text{HO}_2$	4.5 (+2)
R9	$\text{CH}_4 + \text{HO} \rightarrow \text{CH}_3\text{O}_2$	6.6
R10	$\text{C}_2\text{H}_4 + \text{HO} \rightarrow 2\text{CH}_2\text{O} + \text{HO}_2$	1.3 (+4)
R11	$\text{CH}_2\text{O} + \text{HO} \rightarrow \text{CO} + \text{HO}_2$	1.8 (+4)
R12	$\text{CH}_2\text{O} + h\nu \rightarrow \text{CO} + 2\text{HO}_2$	4.3 (-4)
R13	$\text{HO}_2 + \text{NO} \rightarrow \text{NO}_2 + \text{HO}$	1.2 (+4)
R14	$\text{CH}_3\text{O}_2 + \text{NO} \rightarrow \text{CH}_2\text{O} + \text{HO}_2 + \text{NO}_2$	7.8 (+2)
R15	$\text{HO} + \text{NO}_2 \rightarrow \text{HNO}_3$	1.8 (+4)
R16	$\text{HNO}_3 + h\nu \rightarrow \text{HO} + \text{NO}_2$	6.6 (-6)
R17	$\text{HO} + \text{O}_3 \rightarrow \text{HO}_2$	5.7 (+1)
R18	$\text{HO}_2 + \text{O}_3 \rightarrow \text{HO}$	1.0
R19	$2\text{HO}_2 \rightarrow \text{H}_2\text{O}_2$	3.7 (+3)
R20	$\text{H}_2\text{O}_2 + \text{HO} \rightarrow \text{HO}_2$	9.5 (+2)
R21	$\text{H}_2\text{O}_2 + h\nu \rightarrow 2\text{HO}$	3.7 (-5)
R22	$\text{HO}_2 + \text{HO} \rightarrow \text{---}$	4.4 (+4)

Rate coefficients are adapted from Hampson and Garvin⁵ and Chameides⁶ for the following conditions: density = 1.7×10^{19} molecules cm^{-3} ($z = 4$ km), $T = 273$ K, water vapour = 3,000 p.p.m., equinox at 45° N. Numbers in parentheses are powers of 10; units are min^{-1} or $\text{p.p.m.}^{-1} \text{min}^{-1}$.

physical removal pathways. Accordingly, two sets of physical removal rates are considered, one representing relatively long (500 days for NO), the other relatively short (10 days for NO), lifetimes against physical removal. The source strengths of O_3 , CO, C_2H_4 and CH_2O in Table 2 are obtained by distributing the estimated global emissions uniformly (with respect to mixing ratio) throughout the troposphere. The source strength of NO_x is varied as a parameter, with the composition of the emissions being held constant at 95% NO and 5% NO_2 . Only the methane concentration is externally imposed, at 1.6 p.p.m.; all other concentrations are determined by the equilibration of sources and sinks.

Figure 1 shows how the calculated steady state varies with NO_x concentration for a weak physical sink. Photolysis of O_3 (R7) yields HO, which reacts with CO and hydrocarbons (R8, R9, R10) to yield HO_2 and CH_3O_2 , which oxidize NO (R13, R14) to NO_2 , which photolyses (R2) to yield O_3 . In the natural troposphere NO_x is the limiting reactant for the photochemical production of O_3 (ref. 3), and the oxidizing potential of the troposphere increases with increasing NO_x concentration. Because the photostationary ratio of NO_2 to NO increases with O_3 (ref. 7), NO_2 must increase with NO_x as long as O_3 does. As the ratio of NO_2 to CO and hydrocarbons increases, however, so does interdiction of the photochemical chain by R15, a non-productive sink for HO. The NO_2 concentration cannot continue to rise indefinitely without reducing the O_3 yield on O_3 photolysis below break-even. At some point, therefore, O_3 must begin to decrease with further increases in NO_x . Figure 1 shows that a break in fact occurs in the neighbourhood of 1 part in 10^9 (p.p.b.) NO_x . Increases in the NO_x concentration beyond this level produce a decrease in the ozone concentration and an even stronger decrease in free radical concentrations, as the growth of sinks (R13, R15) compounds the decline of the source (R7). Similar calculations by Stewart *et al.*⁸ and Hameed *et al.*⁴ have yielded the same general pattern, with O_3 and HO peaking at slightly lower NO_x concentrations. An analogous nonlinearity in the chlorine chemistry of the stratosphere has been demonstrated by Prather *et al.*⁹ and Fox *et al.*¹⁰.

Figure 2 shows the effect of the break in photochemical reactivity on the NO_x removal rate. As a function of NO_x

Table 2 External source and sink strengths used in calculations

	Source Q (p.p.b. min^{-1})	Sink $1/R$ (days)	
		Weak	Strong
NO	Variable	500	10
NO_2	Variable	50	10
O_3	3×10^{-4}	50	10
CO	7×10^{-4}	500	500
C_2H_4	5×10^{-6}	50	10
CH_2O	6×10^{-7}	50	10
CH_4	Concentration imposed		
HO	0	10	1
HO_2	0	10	1
CH_3O_2	0	10	1
H_2O_2	0	10	1
NO_3	0	10	1
HNO_3	0	10	1

Source strengths of O_3 , CO, C_2H_4 and CH_2O represent estimated global emissions^{6,19,20}, distributed uniformly (with respect to mixing ratio) throughout the troposphere. The source strength of NO_x is varied as a parameter, with the composition of the emissions held constant at 95% NO and 5% NO_2 . The methane concentration is fixed at 1.6 p.p.m. Sink strengths, R , are the first-order coefficients for removal by rainout and other mechanisms not included in the reaction scheme. Two sets of sink strengths are used, presented here in terms of lifetimes, their reciprocals.

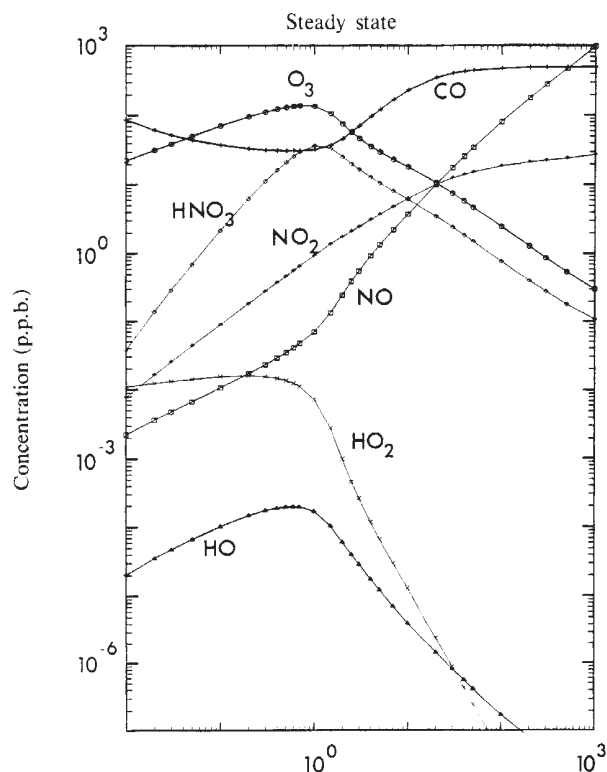


Fig. 1 Calculated steady state of the free troposphere as a function of NO_x ($= \text{NO} + \text{NO}_2$) concentration. Conditions are those of Tables 1 and 2, with a weak physical sink.

concentration, the NO_x sink has a relative maximum where the reactivity peaks, and a relative minimum where oxidation and physical removal are of comparable importance. A similar pattern is observed for a strong physical sink, with the photochemical peak slightly attenuated and the transition to predominantly physical removal occurring at lower NO_x concentration. The effect of holding the CO concentration fixed at 150 p.p.b., shown in Fig. 2 for a weak sink, is to accentuate

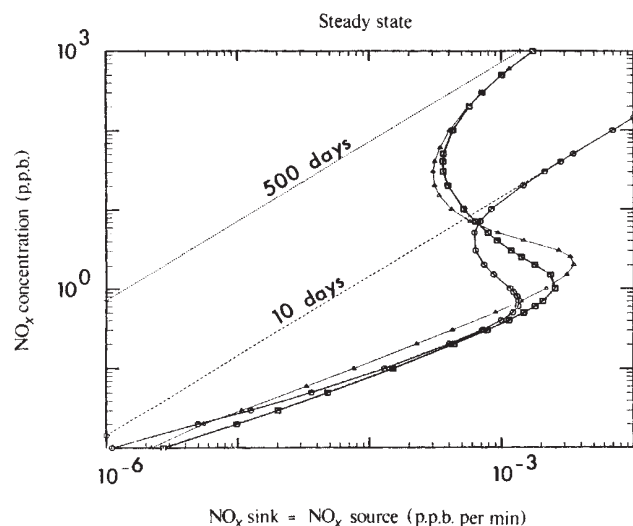


Fig. 2 Calculated steady-state NO_x ($= \text{NO} + \text{NO}_2$) concentration in the free troposphere, as a function of NO_x source strength. The heavy curves are calculated from the reaction scheme of Table 1 and the source strengths of Table 2; the one marked with circles corresponds to the strong physical sink of Table 2, the one marked with squares to the weak physical sink. The light curve (marked with triangles) shows the effect of fixing the CO concentration at 150 p.p.b., for the weak physical sink. The sloping lines, labelled by the lifetime of NO against physical removal, show the linear relationship of concentration to emissions in the absence of photochemical oxidation.

both peak and trough in the sink strength. (The free-floating calculations underestimate the CO concentration, presumably due to gaps in the inventory of emissions (compare refs 6 and 11). The non-monotonic dependence of the NO_x sink on NO_x concentration is, in sum, a fairly robust feature of the calculation. The principal limitation of this result arises from its basis in diurnally averaged photolysis rates, which do not allow for the buildup of NO_3 in the dark. The rapid equilibration of NO_3 with soluble N_2O_5 provides, in the boundary layer and in clouds, a potentially significant night-time pathway for the oxidation and removal of NO_x (refs 2, 12). The calculations presented here are therefore most relevant away from clouds in the free troposphere where, in the absence of liquid water, NO_3 and N_2O_5 simply accumulate overnight and revert to NO_x at day-break.

It is clear from Fig. 2 that dissimilar NO_x concentrations can yield similar NO_x removal rates. As the steady-state NO_x concentration is determined by equilibration of source and sink strengths, this means that a given rate of emission may support more than one steady-state concentration. Consider a source strength for NO_x of 1 part in 10^{12} (p.p.t.) per min, for example, in combination with a strong or weak physical sink. Figure 2 shows that three distinct steady states are possible in either situation. The low- NO_x state is stable, and is characterized by concentrations which are not uncommon over industrial regions: 0.40 p.p.b. NO_x and 64 p.p.b. O_3 for a strong sink, 0.36 p.p.b. NO_x and 120 p.p.b. O_3 for a weak sink. The middle- NO_x state is unstable, and is therefore of no practical interest. The high- NO_x state is characterized by concentrations not presently seen in the free troposphere: 13 p.p.b. NO_x and 4 p.p.b. O_3 for a strong sink, 500 p.p.b. NO_x and 0.5 p.p.b. O_3 for a weak sink. The high- NO_x state is stable, however, and would be expected to persist once established in a sufficiently large region. A shift from the low- NO_x state to the high- NO_x state would profoundly change the reactivity of the troposphere, permitting the accumulation of CO and other trace gases presently scavenged by O_3 and HO (ref. 13). (One of the gases to accumulate would be CH_4 , assumed in our calculation to remain at constant concentration—because of this and other neglected long-term feedbacks, the high- NO_x solution is best viewed as a quasi-steady

state whose ultimate fate is difficult to predict.) An emissions transient capable of shifting a large region into the high- NO_x state is not outside the realm of possibility; Crutzen and Birks¹⁴ calculate that nuclear war could inject of the order of 100 p.p.b. NO_x into the upper troposphere between 40° and 60° N.

In the conditions considered here, multiple steady states become possible at NO_x source strengths above 0.3–0.6 p.p.t. min^{-1} , where the minimum NO_x concentration is ≥ 0.2 p.p.b. Actual NO_x concentrations reach the 0.2 p.p.b. threshold over industrial continents², but tend to be substantially lower over remote areas¹⁵. Actual NO_x emissions are currently estimated² to average about 30×10^9 molecules $\text{cm}^{-2} \text{s}^{-1}$ at 45° N. Much of this originates within the boundary layer, so that the effective source to the free troposphere depends on the rapidity and nature of exchange between the two layers¹⁶. Even if they were distributed uniformly in the vertical, however, such emissions would correspond to a source strength of only 0.1 p.p.t. min^{-1} supporting a unique steady-state NO_x concentration of ~ 0.1 p.p.b. It appears likely, therefore, that current emissions do not quite suffice to support nonstandard steady states on the zonal scale considered here. This is not a firm conclusion, because the viability of alternative steady states is sensitive^{10,17} to details of atmospheric mixing which are poorly understood. It should also be noted that natural emissions of NO_x are rather uncertain², and that anthropogenic emissions are rapidly increasing¹⁸.

Received 5 December 1983; accepted 24 February 1984.

- Schwartz, S. E. & White, W. H. in *Trace Atmospheric Constituents* (ed. Schwartz, S. E.) 1–116 (Wiley, New York, 1983).
- Ehhalt, D. H. & Drummond, J. W. in *Chemistry of the Unpolluted and Polluted Troposphere* (eds Georgii, H. W. & Jaeschke, W.) 219–251 (Reidel, Dordrecht, 1982).
- Fishman, J. & Crutzen, P. J. *Nature* **274**, 855–858 (1978).
- Hameed, S., Pinto, J. P. & Stewart, R. W. *J. geophys. Res.* **84**, 763–768 (1979).
- Hampson, R. F. & Garvin, D., *Reaction Rate and Photochemical Data for Atmospheric Chemistry—1977* (Spec. Publ. 513 NBS, Washington, 1978).
- Chameides, W. L. in *Man's Impact on the Troposphere* (eds Levine, J. S. & Schryer, D. R.) 81–108 (NASA, Washington, 1978).
- Leighton, P. A., *Photochemistry of Air Pollution* (Academic, New York, 1961).
- Stewart, R. W., Hameed, S. & Pinto, J. P. *J. geophys. Res.* **82**, 3134–3140 (1977).
- Prather, M. J., McElroy, M. B., Wofsy, S. C. & Logan, J. A. *Geophys. Res. Lett.* **6**, 163–164 (1979).
- Fox, J. L., Wofsy, S. C., McElroy, M. B. & Prather, M. J. *J. geophys. Res.* **87**, 11126–11132 (1982).
- Crutzen, P. J. & Gidel, L. T. *J. geophys. Res.* **88**, 6641–6661 (1983).
- Richards, L. W. *Atmos. Envir.* **17**, 397–402 (1983).
- Levy, H. II *Science* **173**, 141–143 (1971).
- Crutzen, P. & Birks, J. *Ambio* **11**, 114–125 (1982).
- Noxon, J. F. *J. geophys. Res.* **83**, 3051–3057 (1978).
- Gidel, L. T. *J. geophys. Res.* **88**, 6587–6599 (1983).
- Dudukovic, M. P. *Chem. Engng Sci.* **32**, 985–994 (1977).
- Husar, R. B. & Holloway, J. M. in *Ecological Effects of Acid Deposition* (Report PM 1636) 95–115 (National Swedish Environment Protection Board, Stockholm, 1983).
- Derwent, R. G. & Hov, O. in *Physico-Chemical Behaviour of Atmospheric Pollutants* (eds Versino, B. & Ott, H.) 367–382 (Commission of the European Communities, Brussels, 1980).
- Gidel, L. T. & Shapiro, M. A. *J. geophys. Res.* **85**, 4049–4058 (1980).

Synthesis of marine humic substances from unsaturated lipids

G. R. Harvey, D. A. Boran, S. R. Piotrowicz & C. P. Weisel

National Oceanic and Atmospheric Administration, Atlantic Oceanographic and Meteorological Laboratory, 4301 Rickenbacker Causeway, Miami, Florida 33149, USA

The origin and structure of the dissolved blue-fluorescent yellow organic substances (Gelbstoff) in seawater have long eluded characterization¹. The fact that this class of organic matter, which comprises 10–50% of the organic carbon in the sea², became known as marine humus (fulvic and humic acids) has led to much confusion because of the implied relationship with soil humus³. Based on chemical and spectral studies of oceanic and coastal humus we recently proposed a class structure and a mechanism

by which these marine humic substances could form². The hypothesis was that they are water soluble, aliphatic organic acids formed by the autoxidative cross-linking of two or more unsaturated lipids released into seawater. We have now confirmed that hypothesis by allowing pure marine lipids and a diatom to autoxidize in seawater in the laboratory. We report here that the yellow substances obtained are similar in all respects to marine humus.

The syntheses were conducted in coastal Miami seawater from which the humus was removed by extraction with Amberlite XAD-2 at pH 2 (refs 2, 4, 5): 10 litres of this water were adjusted to pH 8.2 in an open steel container. About 50–100 mg of the particular lipid, dissolved in 5 ml of ethyl ether, were added in 5 mg aliquots to the seawater every 0.5 h until the final concentration was 5 mg l⁻¹. Stirring was continuous throughout the 24–100 h duration of the experiment with the water fully exposed to air and laboratory light. To terminate the synthesis the water was acidified to pH 2 and after 1 h passed through a 75 cm³ column of XAD-2 at 60 ml min⁻¹. Isolation of the products from the resin with methanolic ammonia was by the same process as described elsewhere^{2,4,5}. The pure lipids used were trilinolein, triolein, glyceryl-1,3-dioleate and glyceryl-1-palmitate-2,3-dioleate. The yields were generally greater than 50 mg. Another synthetic, fulvic acid, was made by allowing freeze-dried *Skeletonema costatum*, a marine diatom, to stir in extracted seawater for 1 week. Fully saturated tripalmitin and tristearin produced no product with the properties of marine humus.

The products obtained from the pure unsaturated lipids and the lipids in the diatom⁶ were pale-yellow powders freely soluble in water but insoluble in ether. An elemental analysis of the product from trilinolein was: C, 45.73%; H, 6.71%; N, 0.86%. These values compare well with those of a fulvic acid from the Sargasso Sea⁷. The $\delta^{13}\text{C}$ of this material was -19.7%, which compares well with the isotope values of three marine fulvic acids we isolated from the Gulf of Mexico, that is, -20.6, -21.5 and -22.0%. Interestingly, the $\delta^{13}\text{C}$ of the trilinolein used was -28.1 but we do not know the cause of this large fractionation. Small amounts (3–5 mg) of synthetic humic acids precipitated at pH 2 from the trilinolein reaction mixtures.

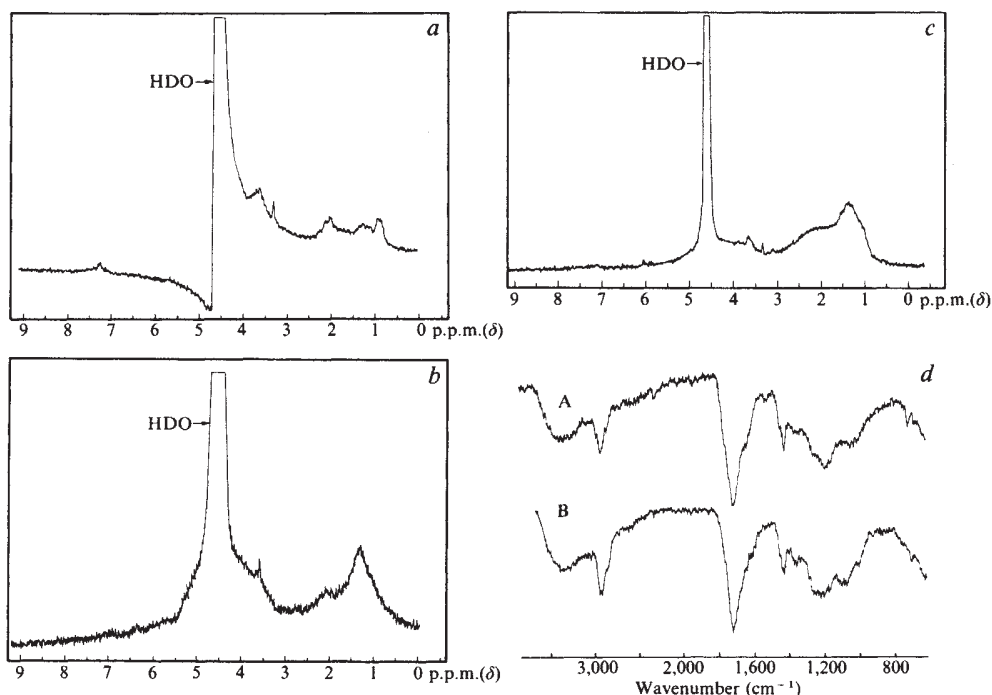
In all cases, the proton NMR spectra of the synthetic humic substances could be matched with one or more spectra obtained from our collection of oceanic humus² (Fig. 1). The extent of cross-linking and heteroatom incorporation can be estimated from the relative widths of the methylene (1–2.5 p.p.m.) and H-C-O- (3–4 p.p.m.) peaks in the NMR. The IR spectrum of

the product of trilinolein autoxidation is superimposable on the spectra of marine fulvics. In addition to the yellow colour of both the natural and synthetic substances, irradiation at 350 nm produces the same broad fluorescence emission between 350 and 650 nm, indicating that the same chromophores are present. Gel permeation chromatography at pH 4, 6 and 8 using both acetate and phosphate buffer eluents (Varian TSK 2000 SW and Sephadex G-25 columns) gave retention times of synthetic and natural fulvics within 5% of each other, indicating similar size and functionality. Although not strictly comparable, polyethylene glycol molecular weight standards indicated a molecular weight range mainly between 600 and 800, which is consistent with our proposed structures. These molecular weight results should be treated with caution because the standards were not the exact same class of compounds⁸.

We have also observed the formation of synthetic marine humic substances by quantifying the development of the zinc complexing ability of seawater containing an unsaturated lipid. We have shown that marine humus is a good complexer of copper and zinc as detected by differential pulse anodic stripping voltammetry (DPASV)^{9,10}. Trilinolein (2.5 mg l⁻¹) was added to aged Gulf Stream water which had little or no complexing capacity for Zn. The Zn signal was monitored periodically for 38 h by standard additions followed by DPASV analysis. The complexing capacity increased from 0.0 at 4 h to 0.5 nmol kg⁻¹ at 8 h to a total of 3 nmol kg⁻¹ at 38 h. The more saturated glyceryl 1,3-dioleate-2-palmitate required 48 h before any increase in Zn complexing capacity was observed. The fully saturated tripalmitin does not autoxidize and did not increase the Zn complexing capacity of the seawater. The different times required by the two lipids to autoxidize and develop Zn complexing ability are related to the number of free radical reactive sites. The synthetic fulvic acid from trilinolein was also shown to protect photosynthetic plankton from copper inhibition to the same degree as a natural marine fulvic¹¹.

The above evidence supports our proposed mechanism of formation and structure of oceanic humus. Humic substances isolated from estuarine and coastal waters are generally admixtures from marine, freshwater and dissolved soil sources and can have different properties from those described for ocean water. The word 'structure' in this context does not refer to a single compound but a closely related family of compounds whose properties can be described by one or two structural formulas as is the case with coal and lignin.

Fig. 1 Proton NMR spectra of the synthetic fulvic acids from trilinolein (a), glyceryl-1,3-dioleate-2-palmitate (b), and an open ocean fulvic acid (c). The IR spectra of the methyl esters of the marine fulvic acid (A) and the trilinolein product (B) are shown in (d). Note the greater amount of unaltered methylene protons (1.2 p.p.m.) in b in comparison with a.



The reactions described here are extracellular examples of the enzyme-controlled cyclization of arachidonic acid (20:4) to prostaglandins¹² and the random free radical autoxidation of cellular unsaturated lipids, a process implicated in many pathologies¹³.

We thank M. Springer-Young and B. Wrenn for laboratory assistance. The carbon isotope data were provided by J. and P. Gearing. The Skeletonema was supplied by R. R. L. Guillard. C.P.W. thanks the US National Research Council for a post-doctoral fellowship. This work was funded by NOAA's Office of Marine Pollution Assessment.

Received 10 November 1983; accepted 8 March 1984.

1. Kalle, K. *Di. Hydrogr. Z.* **2**, 117–124 (1949).
2. Harvey, G. R., Boran, D. A., Chesal, L. A. & Tokar, J. M. *Mar. Chem.* **12**, 119–132 (1983).
3. Pocklington, R. *Mar. Chem.* **5**, 479–496 (1977).
4. Stuermer, D. H. & Harvey, G. R. *Deep-Sea Res.* **24**, 303–309 (1977).
5. Fu, T. & Pocklington, R. *Mar. Chem.* **13**, 255–264 (1983).
6. Ackman, R. G., Jangaard, P. M., Hoyle, R. J. & Brockerhoff, H. *J. Fish. Res. Bd Can.* **21**, 747–756 (1964).
7. Stuermer, D. H. & Harvey, G. R. *Nature* **250**, 480–481 (1974).
8. Thurman, E. M., Wershaw, R. L., Malcolm, R. L. & Pinckney, D. J. *Org. Geochem.* **4**, 27–36 (1982).
9. Piotrowicz, S. R., Springer-Young, M., Puig, J. A. & Spencer, M. J. *Analyt. Chem.* **54**, 1367–1371 (1982).
10. Piotrowicz, S. R., Harvey, G. R., Springer-Young, M., Courant, R. A. & Boran, D. A. in *Trace Metals in Sea Water* (eds Wong, C. S., Boyle, E., Bruland, K., Burton, J. D. & Goldberg, E. D. 699–718 (Plenum, New York, 1983).
11. Ortner, P. B., Kreader, C. & Harvey, G. R. *Nature* **301**, 1–3 (1983).
12. Nelson, N. A., Kelly, R. C. & Johnson, R. A. *Chem. Engng News*, 30–34 (1982).
13. Burton, G. W. & Ingold, K. U. *J. Am. chem. Soc.* **103**, 6472–6477 (1981).

Ciliated protozoa associated with oceanic sinking detritus

Mary Wilcox Silver*, Marcia M. Gowing*,
David C. Brownlee†‡ & John O. Corliss†

* Marine Sciences, University of California, Santa Cruz,
California 95064, USA

† Department of Zoology, University of Maryland, College Park,
Maryland 20742, USA

In the past decade, protozoa have been recognized as ubiquitous residents of the upper waters of the sea, where they have key roles in detrital and producer food webs^{1–4}. In contrast, protozoa are almost unknown from aphotic depths, where food chains presumably are supported by detritus. We show here that ciliate protozoans do accompany sinking detritus to bathypelagic depths. In the productive northeastern Pacific Ocean, the biomass of ciliates on particles settling between the surface and 2,000 m far exceeds the biomass of bacteria, which are the only widely recognized detrital microorganisms in deep pelagic communities. Although absolute numbers of ciliates decline below the euphotic zone, their concentrations (number per g detritus) drop only slightly by 2,000 m. Taxonomic data and patterns of abundance⁵ indicate that the ciliates are endemics and not remnants of surface colonists, and that the deep sea is a favourable environment for them. Their high concentrations, possibly together with other smaller protozoa, indicate that protists may control bacterial mineralization rates on sinking particles, thereby regulating the vertical distribution of many biologically active elements in the sea.

We collected sinking particles in drifting, preservative-filled sediment traps⁶ deployed in the high productivity, eastern boundary current off central California (vertical transport and exchange of materials in the upper waters of the ocean, VERTEX I) and Mexico (VERTEX II and III). Bacteria and ciliates in the traps were counted using methods outlined in Fig. 1. The microorganisms we found were probably associated with the trapped particles, as the sinking rates of individual cells would be insufficient to account for their numbers in the collections.

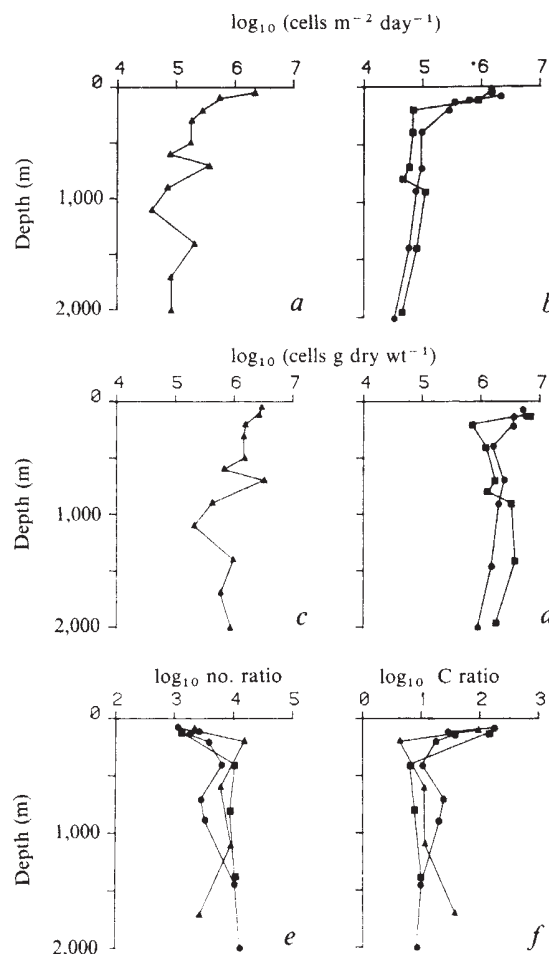


Fig. 1 Microorganisms (numbers and biomass) from sediment traps in the northeastern Pacific Ocean during VERTEX cruises. During VERTEX I (▲), traps were deployed from 26 August 1980 to 8 September 1980 at 35° N; 123° W, during VERTEX II (■) from 27 October 1981 to 17 November 1981 at 17° N; 109° W, and during VERTEX III (●) from 9 November 1982 to 30 November 1982 at 16° N; 107° W. Because they collect for several weeks, traps measure the time-averaged standing crop on sinking particles, or 'flux' of organisms: numbers $\text{m}^{-2} \text{day}^{-1}$. Ciliates in the traps were counted and sizes measured at $\times 400$ in 3–5 aliquots in settling chambers with an inverted phase microscope. Specimen condition was checked in aliquots prepared for transmission electron microscopy (TEM). Protargol silver stained¹³ specimens were identified using oil-immersion, bright-field optics. Bacteria stained with diamidinophenylindole^{14,15} were counted on 0.2 μm Nuclepore filters at $\times 1,188$ with oil immersion optics from three aliquots of trap material and sizes measured in TEM preparations¹⁶. Carbon content was estimated using carbon-volume relationships from the literature^{17,18}. *a*, Ciliate flux during VERTEX I. *b*, Ciliate flux during VERTEX II and III. *c*, Ciliate flux per g dry weight of trap material from VERTEX I. *d*, Ciliate flux per g dry weight of trap material from VERTEX II and III. *e*, Ratio of numbers of bacteria to numbers of ciliates in the trap material from the three cruises. *f*, Ratio of biomass (cell carbon: see above) of ciliates to biomass of bacteria in the trap material from the three cruises.

Ciliates were most common in the shallowest traps, but flux remained above 10^4 cells $\text{m}^{-2} \text{day}^{-1}$ at all depths (Fig. 1*a, b*). The decrease in trap-collected ciliates with depth largely resulted from reduction in the quantity of sinking particulate matter, since numbers per unit dry weight declined only slightly with depth (Fig. 1*c, d*). Ciliate flux showed no consistent reduction in the pronounced oxygen minimum between 600 and 950 m on VERTEX I (ref. 7) and between 100 and 1,000 m on VERTEX II (ref. 8) and III (W. Broenkow, personal communication). Although bacteria outnumbered ciliates by 10^3 – 10^4 (Fig. 1*e*),

‡ Present address: Institute of Marine Resources, A-018, Scripps Institute of Oceanography, La Jolla, California 92093, USA.

Table 1 Ciliates* associated with vertically sinking particles† collected during VERTEX II‡ in the Pacific off central Mexico

Depth	30 m	120 m	1,950 m
Numerical dominants	(1) Oligotrichs (such as <i>Strombidium</i> , <i>Tontonia</i>) (2) Haptorids (such as didiniids)	(1) Scuticociliates (2) Protozoanomatids (such as <i>Metacystis</i>)	(1) Protozoanomatids (such as <i>Metacystis</i>) (2) Scuticociliates (3) Nassulids (4) Oligotrichs (such as <i>Strombidium</i> , <i>Strobilidium</i>)
No. of species per 50 specimens	19	10	15
No. of species shared between adjacent depths		0	5

At our sampling site, temperature at 30 m was 38.5 °C, at 120 m was 14.0 °C, and at 1,950 m was 2.3 °C and water depth was ~3,500 m. Major ciliate orders are listed and species diversity is indicated. Specimens were classified using the taxonomic scheme of Corliss⁹.

* Some fixation-related shrinkage occurred and loss of especially delicate cells was possible; controls for possible cellular lysis in sediment traps are not available. Predeployment checks of net-collected, surface populations with a variety of fixatives, however, indicated our trap preservation solution caused comparatively little ultrastructural (TEM) damage to cells. (Species lost due to fixation could affect the apparent 'endemism' to an unknown extent. Quantitatively, however, losses would result in the underestimation of ciliate importance in our samples.)

† We assume these ciliates represent populations accompanying vertically sinking particles—that significant amounts of allochthonous particulates were not introduced at mid-depth by horizontal advection. A common surface source for the three traps was indicated by their similar phytoplankton debris. Furthermore, current flow measurements did not suggest a physical basis for the largest vertical species change—that found between populations in the two upper traps: the measured maximum current shear⁸ and the predicted sinking speed of marine snow¹⁹ indicate a horizontal displacement of no more than 30 km between sources for the two traps. Most likely, species changes in the living microbial populations resulted from environmental stresses, particularly the low oxygen levels at mid-depth. Moreover, chemical analyses do not indicate significant new inputs of particulate materials at the two deeper trap depths²⁰.

‡ Although we did not perform such a detailed species analysis on materials from VERTEX I or III, a preliminary study of ciliates from the Central California Site (I) showed similar depth-related changes as were observed at the Mexican site (II) and similar ciliate species arrays at depth.

ciliate biomass exceeded that of bacteria due to the relatively large size of the protozoa (usually 10–150 µm compared with <1 µm, respectively). The ciliate dominance was most pronounced near the surface, but ciliate biomass still exceeded that of bacteria in the deepest traps (Fig. 1f).

Taxonomic composition of ciliate populations changed markedly with depth (Table 1). Species richness was highest in the near-surface trap, lowest in the oxygen minimum zone, and intermediate at the greatest depth. The ciliate taxa in the shallowest trap were similar to those from the euphotic zone, but shared no species with the deeper populations. The two deep-water ciliate samples contained taxa not normally reported in lists of planktonic organisms, possessed some of the same species, and included forms that represent not only new species and genera but even higher ciliate taxa (ref. 5 and D.C.B. and J.O.C., manuscript in preparation; also see the classification used in ref. 9).

Our VERTEX results point to several conclusions. First, protozoa on sinking pelagic detritus from the deep sea are as highly concentrated per unit dry weight of detritus as those from any shallow water or laboratory detritus^{10–12}. Furthermore, ciliate concentrations on sinking particles remain high with depth, demonstrating that the decline in ciliate flux is due mostly to the decrease in the quantity of sinking material. The numbers of ciliates, their ultrastructural integrity in electron microscope sections, and their possession of food materials in vacuoles suggest participation in food webs to 2,000 m. The change in species composition with depth further supports the view that the ciliates are not passive members of the rich microbial com-

munities but are populations actively responding to environmental alterations.

The numbers of ciliates relative to bacteria (Fig. 1e) indicate that decomposer communities of the aphotic zone have a population composition resembling that of other detrital communities and thus may be biologically regulated by mechanisms common to the others. In well-established detrital systems, substrate decomposition is largely due to bacteria but protozoa control the rates of mineralization by the bacteria by grazing on them. A variety of protozoa are usually present in detrital communities, but the largest of these—the ciliates—play pivotal roles in structuring the communities: they affect overall decomposition rates by consuming the abundant smaller protozoa that eat bacteria as well as by directly grazing the bacteria^{10–12}. The deep-water ciliates we describe seem to belong to the decomposer assemblage on particulates because they contain bacteria, detritus, and other local food materials in their vacuoles, and no other significant ciliate food resources are available at these depths: the concentrations of cells in the water (as noted from water bottle samples) are several orders of magnitude less than on the sinking particles (unpublished results from this study and ref. 19). The abundance relationships between ciliates and bacteria further argue for the regulatory role of the former: communities with numerical ratios of bacteria/ciliates ranging from 10² to 10⁴ represent detrital systems in which bacterial production is ultimately regulated and mineralization rates stimulated by the ciliates^{10–12}. Since bacterial/ciliate ratios on sinking detritus from both euphotic and aphotic zones fall within this range, an equivalent functional role for the ciliated protozoa seems likely. Such a role does not negate the importance of other, smaller protozoans including microflagellates (which were also present but not counted in the present study), which may be the primary consumers of bacteria, but ciliates are probably the group that ultimately moderates the population sizes and activities of all the lower trophic level members of the decomposer community.

Because ciliates are so much larger than bacteria, the food base that supports the decomposer community, they are available to some consumers unable to capture bacteria. Ciliates may be significant trophic intermediaries between the prokaryotes, which are well known components of deep ocean food webs, and the metazoa. Our studies suggest that the ciliates could have that role on the particulates that rain into the deep sea.

Biological materials on sinking detritus are usually thought to originate from surface waters. However, the ciliates suggest more complex interactions among living components as the particles sink. The marked changes in species composition with depth indicate either a multiple-depth origin of the detritus, a colonization of the detritus at several depths by regional endemics, or a succession in which initially rare species progressively replace the early dominants. The high diversity of the protozoa, furthermore, indicates that the detritus represents a favourable growth environment and that ciliates occupy various niches in these densely populated micro-habitats. Thus the taxonomic data, as well as the biomass measurements, show that sinking particles support active, concentrated, and biologically regulated microbial communities passing through one of the most sparsely populated environments in the biosphere.

We thank G. Knauer and J. Martin for use of the sediment traps; G. Knauer for dry weight flux values; P. Davoll for discussion and help in cruise staging; D. L. Garrison for help with graphics; P. Bolt, J. Mitchell and D. Hurley for help with sectioning; R. Hinegardner, D. Potts, J. Beers and P. M. Holligan for commenting on our manuscript; and the crew of the RV *Wecoma*. The research was funded by NSF-IDOE OCE80-03200.

Received 17 October 1983; accepted 29 February 1984.

1. Beers, J. R. & Stewart, G. L. *Mar. Biol.* 4, 182–189 (1969).
2. Beers, J. R., Reid, F. M. H. & Stewart, G. L. *Mar. Biol.* 60, 209–216 (1980).
3. Sieburth, J. McN. *Helgoländer Wiss. Meeresunters* 30, 697–704 (1977).
4. Azam, F. et al. *Mar. Ecol. Prog. Ser.* 10, 257–263 (1983).
5. Silver, M. W., Brownlee, D. C., Gowing, M. M. & Corliss, J. O. *J. Protozool. Abstr.* 29, 486 (1982).
6. Gowing, M. M. & Silver, M. W. *Mar. Biol.* 73, 7–16 (1983).

7. Broenkow, W. & Greene, N. *Moss Landing Marine Laboratory Tech. Publ.* 81-1 (Moss Landing, California 1981).
8. Broenkow, W. & Krenz, R. *Moss Landing Marine Laboratory Tech. Publ.* 82-1 (1982).
9. Corliss, J. O. *The Ciliated Protozoa: Characterization, Classification, and Guide to the Literature*, 2nd edn (Pergamon, New York, 1979).
10. Fenchel, T. *Limnol. Oceanogr.* 15, 14-20 (1970).
11. Fenchel, T. & Harrison, P. in *The Role of Terrestrial and Aquatic Organisms in Decomposition Processes* (eds Anderson, J. M. & Macfadyen, A.) (Blackwell, Oxford, 1976).
12. Fenchel, T. M. & Jorgensen, B. B. *Adv. Microbiol. Ecol.* 1, 1-57 (1977).
13. Galligher, A. E. & Kozloff, E. N. in *Essentials of Practical Microtechnique* 2nd edn (Lea & Febiger, Philadelphia, 1971).
14. Coleman, A. W. *Limnol. Oceanogr.* 25, 948-951 (1980).
15. Porter, K. G. & Feig, Y. S. *Limnol. Oceanogr.* 25, 943-947 (1980).
16. Silver, M. W. & Bruland, K. W. *Mar. Biol.* 62, 263-273 (1981).
17. Watson, S. J., Novitsky, T. J., Quinby, H. L. & Valois, F. W. *Appl. envir. Microbiol.* 33, 940-946 (1977).
18. Beers, J. R. & Stewart, G. L. *Bull. Scripps Instn Oceanogr. tech. Ser.* 17, 67-87 (1970).
19. Silver, M. W. & Alldredge, A. L. *J. mar. Res.* 39, 501-530 (1981).
20. Martin, J. H., Knauer, G. A. & Gordon, R. M. *Nature* 305, 306-309 (1983).

Remote telemetry of daily vertical and horizontal movement of *Nautilus* in Palau

Peter Ward*, Bruce Carlson†, Michael Weekly‡ & Brian Brumbaugh§

* Department of Geology, University of California, Davis, California 95616, USA

† Waikiki Aquarium, Honolulu, Hawaii

‡ Monterey Aquarium, Monterey, California, USA

§ Hartenbower Sales, Seattle, Washington 98121, USA

Vertical depth migrations, by the chambered cephalopod *Nautilus* when moving into shallower waters at night, were first documented by Wiley¹. Although unsupported by quantitative data, this observation strongly influenced the interpretation of the mode of life of *Nautilus* and of the palaeoecology of fossil chambered cephalopods²⁻⁴. To determine whether these migrations do occur, we mounted ultrasonic transmitters on four *Nautilus belauensis* in Palau. The transmitters contained a strain-gauge depth sensor that caused the transmitters to emit sonic pulses at intervals that varied with depth, so that geographical position and depth could be ascertained. Our results indicate that at least in this population or geographical area, vertical depth changes of up to 200 m per day are common. Although too soon to make generalizations about other populations of *Nautilus* in the Pacific, it has been suggested that the morphological position of the siphuncle within the phragmocone of the chambered shell would increase the efficiency of vertical migrations in *Nautilus*, if such migrations were found to occur^{5,6}. As similar siphuncle configurations are common in most post-Triassic nautiloids, and some ammonoids^{6,7}, it could be that vertical migration was common in many fossil forms as well.

The sonic transmitters were first calibrated in pressure tanks, and by submergence in the sea to known depths. The devices were designed to emit at different frequencies (between 38 and 41 kHz), allowing two *Nautilus* to be tracked simultaneously, and were filled with non-compressible glass microballoons to make the transmitters neutrally buoyant in seawater. The batteries gave the transmitters a life of 7-14 days, and a horizontal range of ~2 km. The transmitters sat in moulded rubber saddles that allowed them to be mounted on the tops of the shells of large, mature *Nautilus* (two males and two females). The *Nautilus* were captured at 200 m in baited traps, and after being fitted with the transmitters, immediately returned to the sea and released by divers at a depth of 70 m. All four *Nautilus* quickly descended to about 200 m, at which point the periods of observation were started. The *Nautilus* were tracked using Dukane hydrophones.

The four *Nautilus* monitored (termed Yellow, Green, Blue and Red, based on colour coding of the transmitters) were tracked for periods of between 2 and 10 days (Figs 1, 2) during June-July 1983. Completeness of tracking depended entirely on weather conditions. Conditions were nearly ideal during the tracking of Yellow, allowing continuous daytime and nighttime

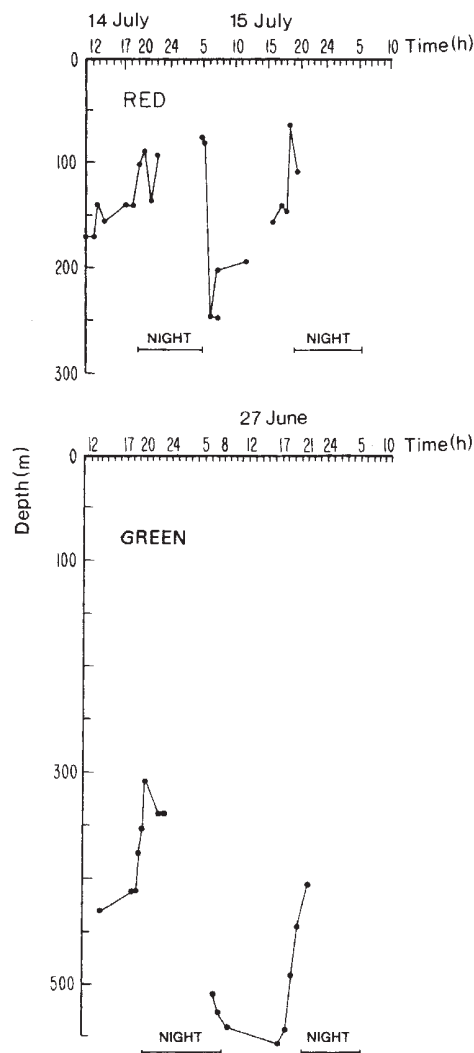


Fig. 1 Vertical movement of two *Nautilus*: Red, followed during 14-15 July 1983, and Green, followed during 27-28 June 1983.

work, but much less favourable for Green, Blue and Red, which were tracked mainly during daytime hours. All four animals showed significantly deeper mean daytime than nighttime depths (Table 1). Yellow, Red and Green showed daily ascents, occurring between 1600 and 2000, and descents between 0400 and 0700. (During our observation period (June-July 1983) sunset occurred at 1815 and sunrise at 0545.) Yellow was extremely regular in its diurnal movements. Blue was more irregular, and on three occasions (8, 10 and 13 October) showed evening descent rather than ascent. Because tracking was incomplete we do not know whether this animal ascended later on these evenings.

The tracking data indicate no daily rest periods. Although the largest scale depth displacements occurred at dawn and dusk, other vertical movements also occurred during day and night-time hours. We never observed any *Nautilus* spending longer than a few hours at the same depth; our data suggest nearly constant activity.

All four *Nautilus* showed substantial horizontal as well as vertical movement. One (Yellow) showed a somewhat regular pattern of movement around the south-west corner of Angulpelu Reef; each daylight period it moved into deeper water on the south side of the reef, while at night it moved around the point, into the large bay defined by Angulpelu and Ngadarak reefs, a distance of ~1 km. The other three *Nautilus* showed a different pattern. After being released near the point of Angulpelu reef, Green, Blue and Red began a daily southwestern movement along the 200 m contour of the bay. Superimposed

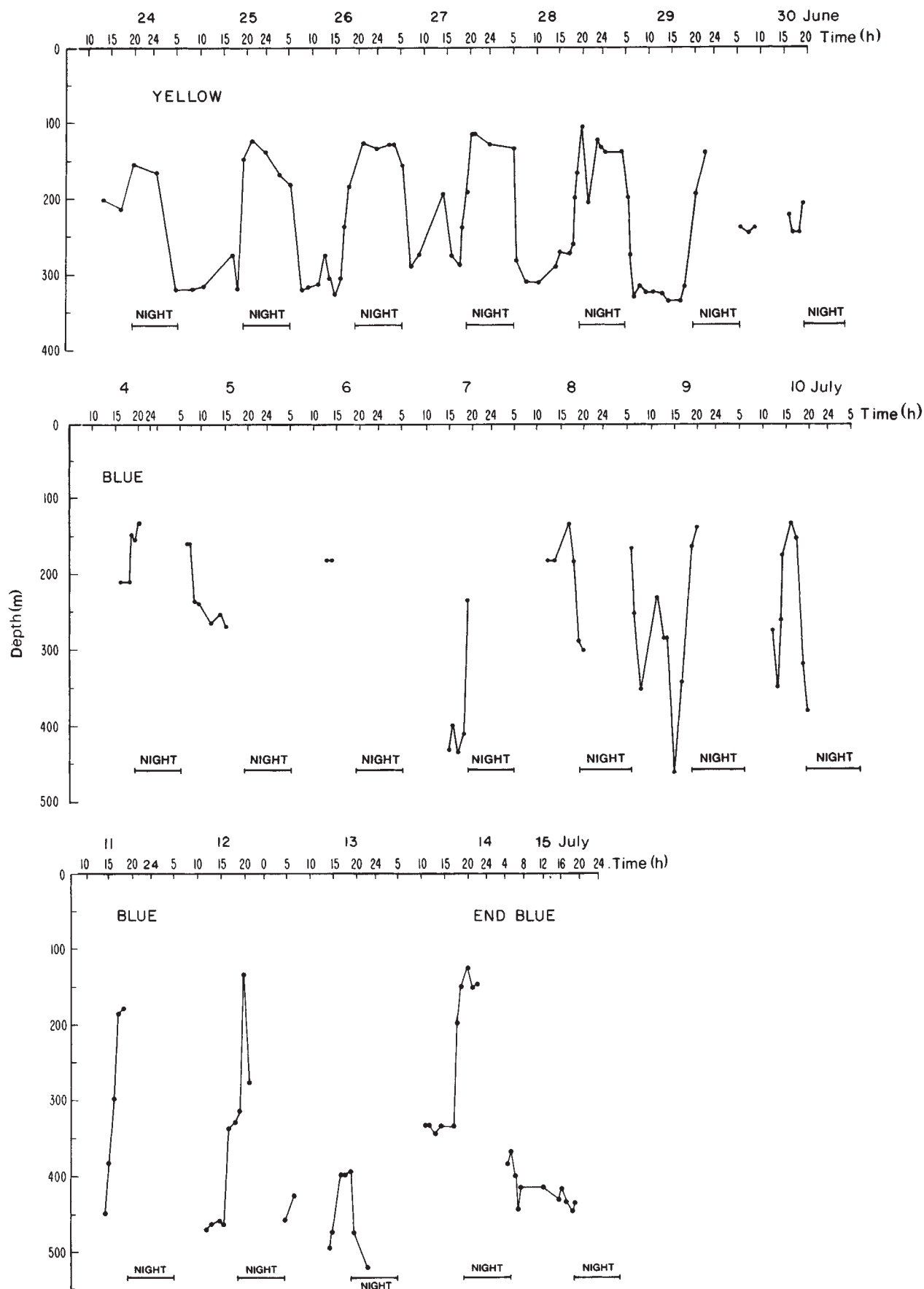


Fig. 2 Vertical movement with time of two *Nautilus belauensis*, Yellow and Blue. The ordinate records the depth, as derived from the strain-gauge depth sensor of the transmitter. Yellow was tracked from 24 to 30 June 1983. Blue was tracked between 4 and 14 July 1983. Each dot represents the mean of 6–10 depth readings taken over a 2–3 min period. Numbers on the horizontal axes refer to the hour of day. Periods of darkness (night) are indicated by bars at the bottom of each graph.

Table 1 Results of depth tracking for four specimens of *Nautilus*

Name	Sex	Dates of observation	Time	Mean day depth† (m)	Mean night depth† (m)
Yellow	M	24–30 June 1983	152	290	153
Blue	F	4–15 July 1983	266	329	260
Green	M	26–27 June 1983	34	441	321
Red	F	7–15 July 1983	32	120	87

* Hours under observation.

† Derived from daytime and nighttime depth figures, with a maximum of two readings during any single hour being included. In the case of Yellow the mean depth figures were computed by integrating the depth curves.

on this southwestern horizontal movement was vertical movement, which was accomplished by inshore and offshore motion. Because of the steepness of the Palauan reefs, very short horizontal distances resulted in large vertical displacements. The Blue animal, followed for the longest period, travelled a total of 16 km along the reef front during the 10-day observation period. Horizontal movements of a similar magnitude have been observed previously for longer time periods in this population of *Nautilus*⁸.

The depths at which *Nautilus* were found, as computed from the ultrasonic emissions, were compared with the bottom depth recorded nine times using a fathometer. The two depths differed significantly in only two cases, both involving the Blue transmitter. On 7 October 1983 Blue was followed for about 6 h as it ascended from 250 m depth to 150 m. At the end of this period the animal resettled to the bottom, after having travelled ~1 km horizontal distance. Although *Nautilus* appear to move mainly on the bottom, there does seem to be some movement within the water column.

We thank James McKibben for construction of the transmitters. This work was carried out at the Marine Mariculture Demonstration Center, Palau. Mr Tosh Paulis helped with boat acquisition. This work was supported by NSF grant PCM 8202891.

Received 19 September; accepted 13 February 1984.

1. Wiley, A. *Proc. zool. Soc. Lond.* 7–9 (1899); *Zoological Results* Part IV, 750–830 (Cambridge University Press, 1902).
2. Stenzel, H. *Treatise on Invertebrate Paleontology*, (ed. Moore, R. C.) 59–93 (University of Kansas Press, 1964).
3. Heptonstall, W. *Lethaia* 3, 317–328 (1970).
4. Mutvei, H. & Reymont, R. *Paleontology* 16, 623–636.
5. Denton, E. & Gilpin-Brown, J. *Adv. mar. Biol.* 11, 197–268 (1973).
6. Ward, P. *Paleobiology* 6, 32–43 (1980).
7. Ward, P. *Paleobiology* 5, 415–422 (1979).
8. Saunders, W. B. & Spinosa, C. *Science* 204, 1199–1201 (1979).

Gradual phyletic evolution at the generic level in early Eocene omomyid primates

Kenneth D. Rose

Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA

Thomas M. Bown

Paleontology-Stratigraphy Branch, US Geological Survey, Denver, Colorado 80225, USA

Analysis of dental morphology in over 600 stratigraphically controlled specimens of tarsier-like primates from early Eocene strata in the Bighorn Basin, Wyoming, provides important new data for understanding the tempo and mode of evolution in primates. Here we describe two evolutionary transitions at the generic level in separate lineages of the family Omomyidae. In both lines transformation occurred not only continuously (rather

than by abrupt appearance of new morphologies followed by stasis), but also in mosaic fashion, with greater variation in certain characters preceding a shift to another character state. In one lineage this resulted in a significant change in dental adaptation. These data support a gradual model of dental evolution and are inconsistent with the punctuated equilibria model. Together with other reported cases, the data suggest that gradualism is much more prevalent than proponents of punctuated equilibria have argued.

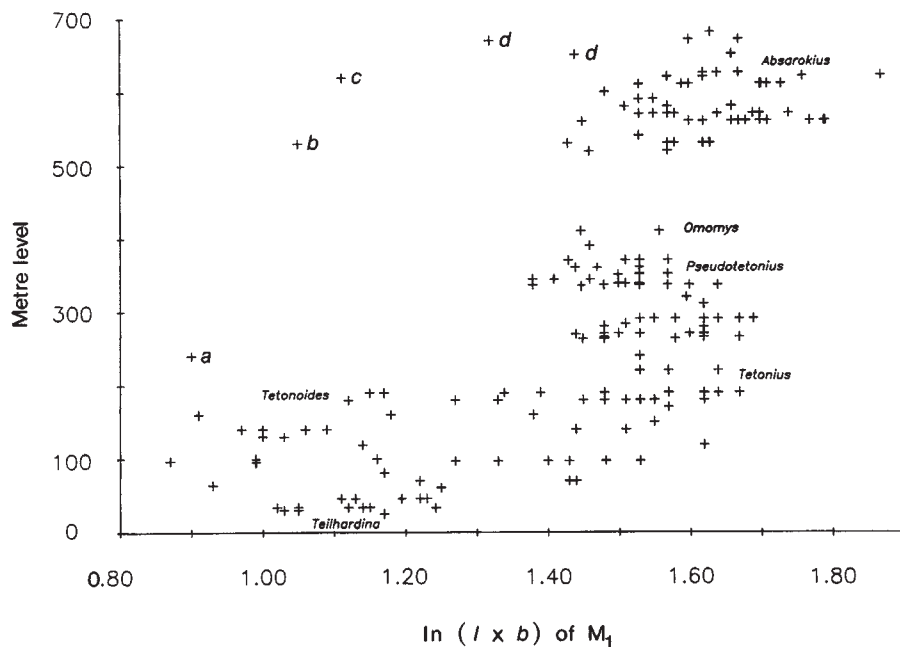
The early Eocene sequence of the Willwood Formation in the Bighorn Basin, Wyoming, is more than 700 m thick, spans about 4 Myr and is among the richest areas for fossil vertebrates in the world. Because of excellent stratigraphical control, samples from these deposits constitute some of the most important data for studies of mammalian evolution^{1–12}, including the largest and most diverse collection of omomyid primates known from any basin. This collection is now several times larger than the largest sample studied by previous workers, and nearly all specimens are tied into measured stratigraphical sections.

The omomyid samples have been assigned to at least six species in as many as six genera: *Teilhardina americana*, *Tetonoides tenuiculus*, *Tetonius homunculus*, *Pseudotetonius ambiguus*, *Absarokius abbotti* and *Omomys vespertinus*^{7,13–20}, and several undescribed species (Fig. 1). The systematics of these fossils is still under study, and it is clear that previous understanding of most of them was very incomplete. Our studies indicate that all of these species are valid, but it is debatable how many genera are represented. Here we provide evidence for the transitions between *Tetonius homunculus* and *Pseudotetonius ambiguus* and between *Teil. americana* and *Tetonoides tenuiculus*. Detailed justification will be provided elsewhere for recognition of *Teil. americana* and *P. ambiguus*, the validity of both of which has been questioned^{20–22}, but for this report, the taxonomy of the fossils is of less consequence than the evolutionary patterns they show.

All omomyids from the Bighorn Basin are conservative in molar morphology, differing consistently only in absolute size or relative breadth. Figure 1 shows that the molars separate to some extent on size alone. *Teilhardina* and *Tetonoides* are about the same size and are significantly smaller than *Tetonius* or *Pseudotetonius*, whereas the latter and *Omomys* are slightly smaller than *Tetonius*, and *Absarokius* is somewhat larger than *Tetonius*. Even so, the latter four taxa overlap substantially in molar size. To distinguish between them, differences in crown morphology, size and configuration of antemolar teeth are more important than molar size. Within each size group (Fig. 1), different morphologies are recognized under the specific names listed above, and our studies demonstrate that each comes from a restricted stratigraphical interval. Precise stratigraphical documentation has, therefore, been indispensable in determining relationships between species.

In the lineage from *Tetonius homunculus* to *P. ambiguus*, the anterior dentition evolved dramatically, while the molars and the last lower premolar (P_4) remained conservative. Here we apply the name *Tetonius homunculus* to the most primitive specimens (see also ref. 19), which have a relatively large, two-rooted P_3 , a very small, one-rooted P_2 , a moderate sized canine and second incisor (I_2), and an enlarged medial incisor (I_1). All such specimens (stage 1 in Fig. 2) are confined to the 180–190 m interval of the Willwood Formation in the principal study area (central and southern Bighorn Basin). *Tetonius* first appears at about 65 m but its anterior dentition is unknown below 180 m. Not all specimens in this lineage possess P_2 ; but from the stratigraphy it became clear that P_2 was lost after stage 1, beginning a trend toward progressive compaction and reduction of anterior teeth, except for I_1 which became larger with time. The name *Tetonius ambiguus* was first applied to a specimen showing extreme compression of the anterior dentition¹⁷, but with increasing knowledge of variation in samples allocated to *Tetonius*, *Tetonius ambiguus* was transferred to a new genus, *Pseudotetonius*¹⁴. *P. ambiguus*, here shown to be the end point of the lineage, is characterized by a much reduced P_3 with one

Fig. 1 Stratigraphical distributions of omomyid primates in the central and southern Bighorn Basin (Elk Creek, Fifteen-mile Creek and Sand Creek sections). Abscissa show the natural logarithm of crown area (mm^2) of the first lower molar; the ordinate represents the stratigraphical level in Willwood Formation. Some points represent more than one individual. Names are placed near clusters of specimens that can be referred on morphological grounds to the species listed in the text and points situated stratigraphically between them represent evolutionary intermediates. Points *a-d* denote individuals belonging to undescribed taxa. Some taxa overlap considerably in size range of M_1 , but their means may differ: for example, mean area of M_1 in *P. ambiguus* is less than in *Tetonius homunculus* and both are smaller than the mean for *A. abbotti*. Plots for other teeth show a similar pattern except that those based on P_3 and M_2 show greater separation between *Tetonius homunculus* and *P. ambiguus*, and that based on P_4 segregates *A. abbotti* more from other samples.



root or coalesced roots, absence of P_2 , diminutive canine and I_2 , and much enlarged I_1 (stages 4 and 5 in Fig. 2). The dentary anterior to P_4 is much shorter than in *Tetonius homunculus*. This morphology occurs in the 350–390 m interval in the study area, at the top of which *P. ambiguus* apparently became extinct (coinciding with an extinction-immigration event termed biohorizon B²³). Intervening strata have revealed morphologically intermediate specimens that record a gradual transition from *Tetonius homunculus* to *P. ambiguus* (Fig. 2).

Of particular interest is the mosaic pattern of change. Modi-

fications that characterize *Pseudotetonius* did not appear synchronously, and certain characters (for example, P_3 root configuration) were more variable during periods of change. P_2 disappeared between stages 1 and 2; P_3 gradually diminished in size throughout the sequence while its two roots became closely appressed and merged into one from stages 2 to 5; the canine and I_2 became gradually smaller up-section; and I_1 enlarged, principally in stages 3 to 5. There was also a slight reduction in mean lengths of P_4 and the lower molars in stages 3–5. The sum of changes during this transition reflect an adaptive

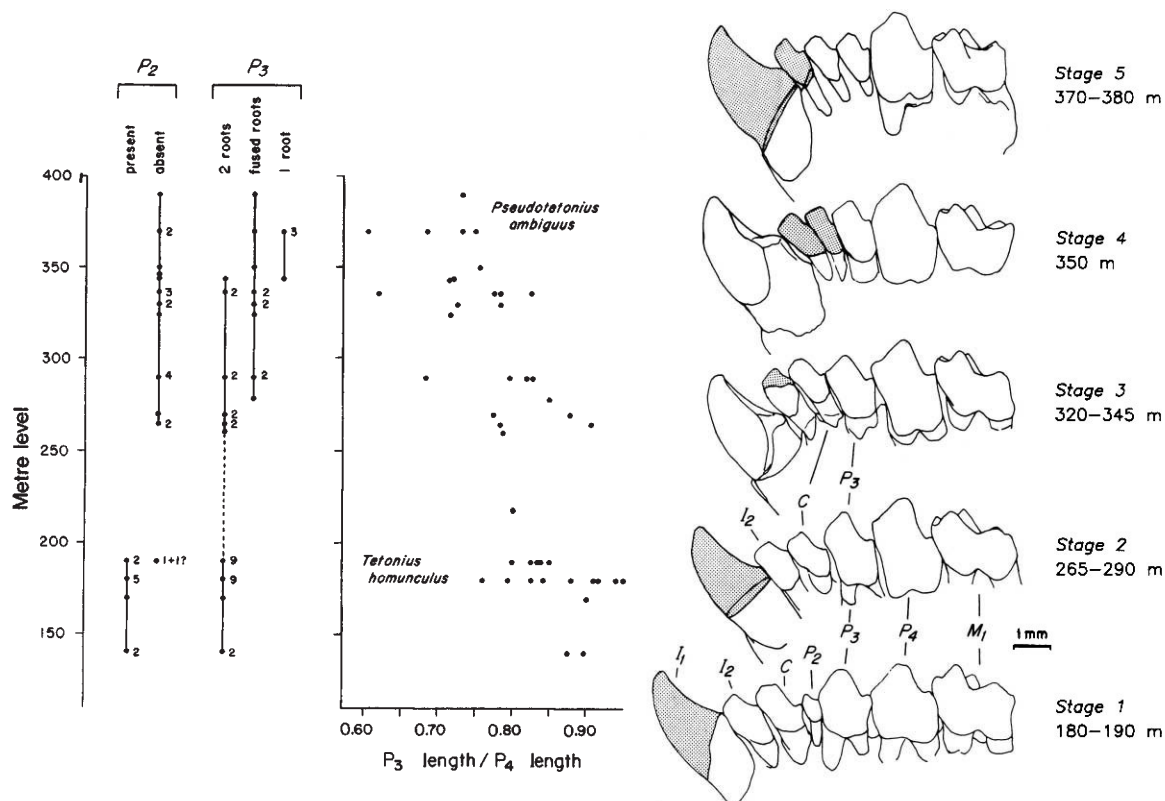


Fig. 2 Evolution of the anterior lower dentition in the *Tetonius homunculus*–*P. ambiguus* lineage from the central and southern Bighorn Basin. Stages in the transition are arbitrarily delimited by slight morphological differences and/or intervals with little or no data, and each is a composite dentition (M_{2-3} omitted) based on all known material from the indicated interval. Shaded parts are reconstructed from root size and known structure in other stages. The stratigraphical distribution of certain character states is shown on the left; numbers of individuals are indicated except where data points represent single specimens.

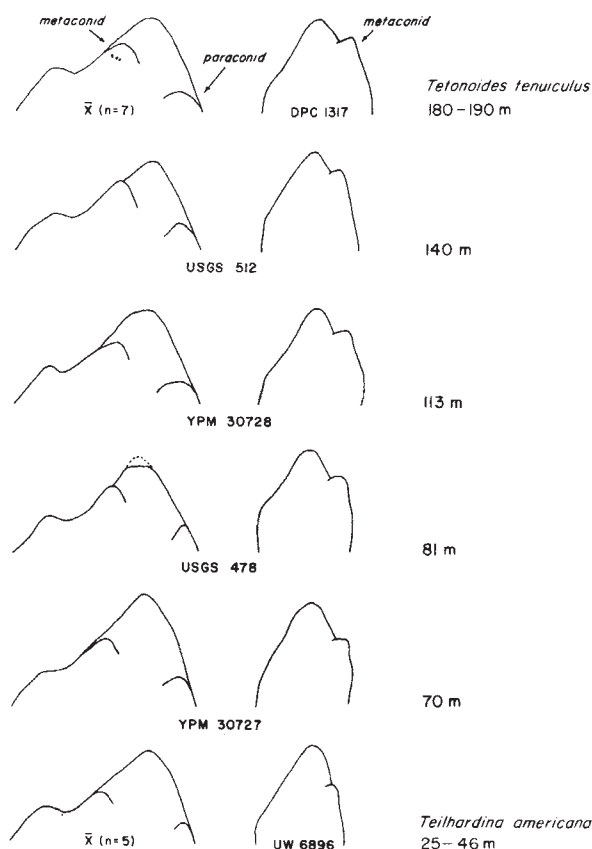


Fig. 3 Transformation of lower fourth premolar (P_4) in the *Teilhardina americana*-*Tetonoides tenuiculus* lineage. Left, computer-generated digitized lingual profiles, standardized to the same length so as to indicate only structural differences. Note the progressive enlargement and elevation of the metaconid and the elongation of the paraconid in successively higher strata. Right, camera lucida tracings, at constant magnification, of the back of the P_4 trigonid, showing the increase in size and height of the metaconid as well as increase in absolute breadth. Profiles for *Teilhardina americana* and *Tetonoides tenuiculus* are means computed from only complete, unworn specimens; other profiles depict individual specimens. Other known specimens from these levels conform to the morphologies shown.

shift to shorter-jawed omomyids with enhanced emphasis of the anterior grasping teeth and declining importance of the teeth just behind them.

Also found is a gradual transition from *Teilhardina americana* to *Tetonoides tenuiculus*, best documented in the morphology of the posterior lower premolars (P_{3-4}). *Teilhardina americana* is the oldest and most conservative North American omomyid^{13,24} and, as in European *Teilhardina belgica* [clearly its congener (K.D.R., T.M.B. and M. Godinot, in preparation)], the P_3 and P_4 are relatively high-crowned, pointed and bear small, low conids, which are more pronounced on P_4 ; the cheek teeth are not basally inflated. These features occur only in the lowest 50 m (25–50 m interval) of the study area. *Tetonoides tenuiculus* differs from *Teilhardina* chiefly in having relatively broader and lower cheek teeth and a more molariform P_4 , best illustrated by the higher, more salient paraconid and metaconid. Specimens in which this morphology is fully developed come from the 180–190 m interval in the study area. Fossils from intervening strata are intermediate between the two morphologies and become progressively more like *Tetonoides* up-section, the cheek teeth increasing in relative breadth and the paraconid and metaconid increasing in prominence with time (Fig. 3). At

no level in the section is there an obvious morphological shift (punctuation); rather, change appears to be continuous and gradual. *Tetonoides tenuiculus* was short-lived in the Bighorn Basin (but a few stratigraphically higher specimens may be related to it: Fig. 1a–c), although the genus is known from younger rocks in other basins^{25,26}.

Both of the phyletic transitions described here are also recorded in samples from the Clark's Fork Basin, 100 km north of the study area. Those samples show the same sequence of changes in both lineages as observed in the study area, thus providing independent confirmation of the patterns reported here.

The tempo and mode of evolutionary change continue to be among the most actively debated issues in evolutionary biology, with particular attention currently focused on punctuational and gradual models of evolution^{3,11,12,27–35}. The most convincing evidence for gradual evolution in fossil mammals has been advanced by Gingerich and colleagues^{1–6}, but these studies concentrate primarily on changes in molar size, and advocates of the punctuated equilibria model largely discount them^{11,12}. The transitions in two lineages of omomyid primates detailed here involve a mosaic of continuously changing features that document the gradual evolution of new morphological types of generic rank. Together with Gingerich's examples and other detailed cases of gradual evolution^{36–38}, our data suggest that phyletic gradualism is not only more common than some would admit but also capable of producing significant adaptive modifications.

For access to specimens and casts used in this study we thank D. Baird, R. T. Bakker, M. R. Dawson, R. J. Emry, P. D. Gingerich, M. Godinot, F. A. Jenkins Jr, J. A. Lillegraven, L. D. Martin, M. C. McKenna, G. E. Meyer, J. H. Ostrom, D. E. Savage, C. R. Schaff and E. L. Simons. We thank A. C. Walker and S. B. Walker for guidance with computer analyses; and P. D. Gingerich, D. W. Krause and A. C. Walker for suggestions. We acknowledge support of this research from the American Philosophical Society (Penrose 9269), National Geographic Society (2366–81), and NSF (BSR-8215099) to K.D.R.

Received 28 November 1983; accepted 23 February 1984.

- Gingerich, P. D. *Nature* **248**, 107–109 (1974).
- Gingerich, P. D. *Am. J. Sci.* **276**, 1–28 (1976).
- Gingerich, P. D. *A. Rev. Earth planet. Sci.* **8**, 407–424 (1980).
- Gingerich, P. D. & Simons, E. L. *Contr. Mus. Paleont. Univ. Mich.* **24**, 245–279 (1977).
- Gingerich, P. D. & Gunnell, G. F. *Contr. Mus. Paleont. Univ. Mich.* **25**, 125–153 (1979).
- Bookstein, F. L., Gingerich, P. D. & Kluge, A. G. *Paleobiology* **4**, 120–134 (1978).
- Bown, T. M. *Mem. geol. Surv. Wyo.* **2**, 1–151 (1979).
- Bown, T. M. & Kihm, A. J. *J. Paleont.* **55**, 257–270 (1981).
- Rose, K. D. *Univ. Mich. Pap. Paleont.* **26**, 1–197 (1981).
- Schankler, D. M. *Nature* **293**, 135–138 (1981).
- Gould, S. J. & Eldredge, N. *Paleobiology* **3**, 115–151 (1977).
- Stanley, S. M. *Macroevolution* (Freeman, San Francisco, 1979).
- Bown, T. M. *Folia primatol.* **25**, 62–72 (1976).
- Bown, T. M. *Univ. Wyo. Contr. Geol.* **13**, 19–26 (1974).
- Jepsen, G. L. *Proc. Am. phil. Soc.* **69**, 117–131 (1930).
- Cope, E. D. *Proc. Am. phil. Soc.* **20**, 139–197 (1882).
- Matthew, W. D. *Bull. Am. Mus. nat. Hist.* **34**, 429–483 (1915).
- Simons, E. L. *Primate Evolution* (Macmillan, New York, 1972).
- Szalay, F. S. *Bull. Am. Mus. nat. Hist.* **156**, 157–450 (1976).
- Gingerich, P. D. *J. hum. Evol.* **10**, 345–374 (1981).
- Szalay, F. S. & Delson, E. *Evolutionary History of the Primates* (Academic, New York, 1979).
- Szalay, F. S. *Folia primatol.* **37**, 153–162 (1982).
- Schankler, D. M. *Univ. Mich. Pap. Paleont.* **24**, 99–114 (1980).
- Savage, D. E., Russell, D. E. & Waters, B. T. *Géobios Mém. spéc.* **1**, 159–164 (1977).
- Gazin, C. L. *Smithson. misc. Collns* **144**, no. 1, 1–98 (1962).
- Savage, D. E., Waters, B. T. & Hutchison, J. H. in *Field Conference on Tertiary Biostratigraphy of Southern and Western Wyoming* (ed. West, R. M.) (1972).
- Eldredge, N. & Gould, S. J. in *Models in Paleobiology* (ed. Schopf, T. J. M.) (Freeman, San Francisco, 1972).
- Vrba, E. S. *S. Afr. J. Sci.* **76**, 61–84 (1980).
- Stebbins, G. L. *Evolution* **36**, 1109–1118 (1982).
- Mayr, E. *Evolution* **36**, 1119–1132 (1982).
- Schopf, T. J. M. *Evolution* **36**, 1144–1157 (1982).
- Levinton, J. S. & Simon, C. M. *Syst. Zool.* **29**, 130–142 (1980).
- Williamson, P. G. *Nature* **293**, 437–443 (1981).
- Stanley, S. M. *Evolution* **36**, 460–473 (1982).
- Charlesworth, B., Lande, R. & Slatkin, M. *Evolution* **36**, 474–498 (1982).
- Ozawa, T. *Mem. Fac. Sci. Kyushu Univ.*, ser. D. **23**, 117–164 (1975).
- Dzik, J. & Trammer, J. *Acta palaeont. pol.* **25**, 55–89 (1980).
- Malmgren, B. A. & Kennett, J. P. *Paleobiology* **7**, 230–240 (1981).

Close genetic linkage between X-linked retinitis pigmentosa and a restriction fragment length polymorphism identified by recombinant DNA probe L1.28

S. S. Bhattacharya*†, A. F. Wright*, J. F. Clayton*, W. H. Price*†, C. I. Phillips‡, C. M. E. McKeown§, M. Jay||, A. C. Bird||, P. L. Pearson¶, E. M. Southern*# & H. J. Evans*

* MRC Clinical and Population Cytogenetics Unit and † University Department of Medicine, Western General Hospital, Edinburgh EH4 2XU, UK

‡ Department of Ophthalmology, University of Edinburgh, Eye Pavilion, Edinburgh EH3 9HA, UK

§ Department of Clinical Genetics, Birmingham Maternity Hospital, Birmingham B15 2TG, UK

|| Department of Clinical Ophthalmology, University of London, Institute of Ophthalmology, London WC1H 9QS, UK

¶ Department of Human Genetics, University of Leiden, The Netherlands

MRC Mammalian Genome Unit, Department of Zoology, Edinburgh EH9 3JT, UK

Retinitis pigmentosa (RP) is a group of retinal degenerations characterized by progressive visual field loss, night blindness and pigmentary retinopathy¹. Its prevalence is in the region of 1–2 in 5,000 of the general population, making it one of the commoner causes of blindness in early and middle life^{2,3}. Although 36–48% of RP patients are isolated cases, the remainder show autosomal dominant, autosomal recessive or X-linked modes of inheritance^{4,5}. The X-linked variety (XLRP) is found in 14–22% of RP families in the UK^{2,5}. In the present study, X chromosome-specific recombinant DNA probes which can detect restriction fragment length polymorphisms have been used to localize the XLRP gene(s) to a subregion of the X chromosome using linkage analysis. One of the probes, L1.28, has been shown to be closely linked to XLRP in five kindreds, with 95% confidence limits of 0–15 centimorgans (maximum LOD score of 7.89 at a distance of 3 centimorgans). This suggests that the XLRP locus lies on the proximal part of the short arm of the X chromosome. This probe is potentially useful for carrier detection and early diagnosis in about 40% of cases, provided that genetic heterogeneity can be excluded by analysis of further families.

XLRP is one of the most severe clinical forms of RP, with onset in males in the first decade, progressing to blindness by the third or fourth decade⁶. Female carriers show a variable but generally mild degree of visual loss, which occurs much later than in males and is associated with characteristically mild ophthalmoscopic changes in the retinal fundus and abnormalities on visual field, psychophysical and electrophysiological testing^{7–9}. Partial manifestation in female carriers is generally attributed to random X-chromosome inactivation leading to unmodified expression of the XLRP allele in a proportion of retinal cells. Clinical and electrophysiological testing may detect only a proportion of carriers and then only at a relatively late stage of the disease. The loci for the Xg blood group and deutan colour blindness are not closely linked to XLRP and are therefore not useful in carrier detection^{10,11}. Recently, restriction fragment length polymorphisms (RFLPs) have extended the utility of polymorphic markers in the detection of genes responsible for genetic disorders such as Huntington's chorea and Duchenne muscular dystrophy^{12,13}.

Families with XLRP were ascertained through ophthalmological genetic clinics in Edinburgh, Birmingham and London. The criteria used for diagnosis and establishment of X-linkage have been described previously¹⁴. Females at risk showing the ophthalmoscopic changes of carrier status, which were con-

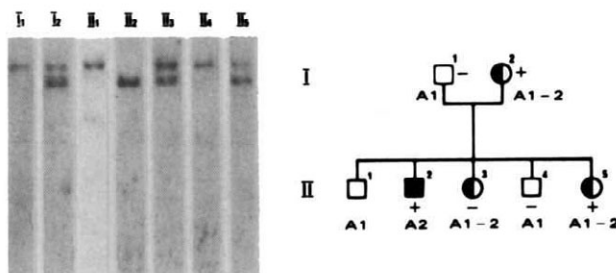


Fig. 1 The segregation of RFLP alleles identified with probe L1.28 in an XLRP family (F21). Carrier females (●) are shown in generations I (I.2) and II (II.3, 5). The mother (I.2) is an obligate heterozygote as she has a severely affected son (II.3, ■) and two carrier daughters. She is also heterozygous for the L1.28 polymorphism, showing the upper (12 kb) and lower (9 kb) bands. The unaffected sons (II.1, 4) have inherited allele A1 (12 kb) while the affected son has inherited allele A2 (9 kb) from the mother, as have the two carrier daughters. Xg^a(+) and Xg^a(-) blood group alleles are also shown.

Methods: DNA was prepared from 20 ml of whole blood or lymphoblastoid cell lines by the method of Kunkel *et al.*²² and digested to completion using 5 units of *Taq*I per μ g DNA for 20 min at 65 °C. The DNA digests (5 μ g per track) were separated by overnight gel electrophoresis in 0.8% agarose, transferred to a nitrocellulose filter by the method of Southern²³ and hybridized for 18 h at 65 °C in 15 ml of 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.1% SDS and 100 μ g ml⁻¹ denatured salmon sperm DNA. The L1.28 plasmid DNA was labelled by nick-translation with ³²P-dTTP (specific activity $\sim 2 \times 10^8$ c.p.m. μ g⁻¹), without excising the insert. The probe concentration in each filter hybridization was 10 ng ml⁻¹ (3×10^7 c.p.m.). The filters were washed at room temperature in 2 $\times$ SSC for 20 min, followed by three washes of 20 min each in 0.1 $\times$ SSC, 0.1% SDS at 60 °C, then autoradiographed on Fuji film at -70 °C for 3–7 days using an intensifier screen.

Table 1 Analysis of linkage between XLRP, Xg and L1.28

Recombination fraction, Θ	XLRP -F15 <i>n</i> = 4	L1.28 F15 alone <i>n</i> = 1	XLRP +F15 <i>n</i> = 5	Xg XLRP +F15 <i>n</i> = 8
0.00	3.45	—∞	—∞	—∞
0.05	3.15	4.65	7.79	-6.81
0.10	2.82	4.45	7.27	-4.00
0.20	2.11	3.69	5.80	-1.65
0.30	1.33	2.69	4.02	-0.63
0.40	0.57	1.47	2.03	-0.16
$\Theta_{\max}$	0.00	0.04	0.03	0.50
LOD _{max} *	3.45	4.65	7.89	0.00
Θ_{mean}	0.08	0.10	0.06	0.47
Range (cM)	0–27	2–25	0–15	38–200
Odds ($\Theta < 0.3$)	79:1	187:1	15,000:1	0.019:1
Odds ($\Theta < 0.2$)	20:1	19:1	400:1	0.001:1

LOD scores are shown for different values of the recombination fraction (Θ) in XLRP families excluding F15 (-F15), including F15 (+F15) and in F15 alone. Maximum likelihood values of Θ ($\Theta_{\max}$) and the corresponding LOD scores (LOD_{max}) are shown together with the mean Θ values, the latter being a value more appropriate to genetic counselling²⁰. A probability distribution for the recombination fraction was calculated from the likelihood curve (calculated at 51 values of Θ) and the linear prior probability for two unknown loci²¹. The Kosambi mapping function was used and the X chromosome was assumed to be 200 centimorgans long. The 95% confidence limits were estimated by excluding 2.5% of the area under the curve at each tail. The 'odds of linkage' were estimated by comparing the area under the curve at values of Θ less than 0.3 or 0.2 with that under the remainder of the curve. The number of informative kindreds is indicated (*n*).

* The LOD score is the $\log_{10}$ of the likelihood ratio, which is calculated by dividing the likelihood of observing the family data, for any value of Θ , by the likelihood if Θ is 0.5 (that is, random assortment or infinite separation of two loci).

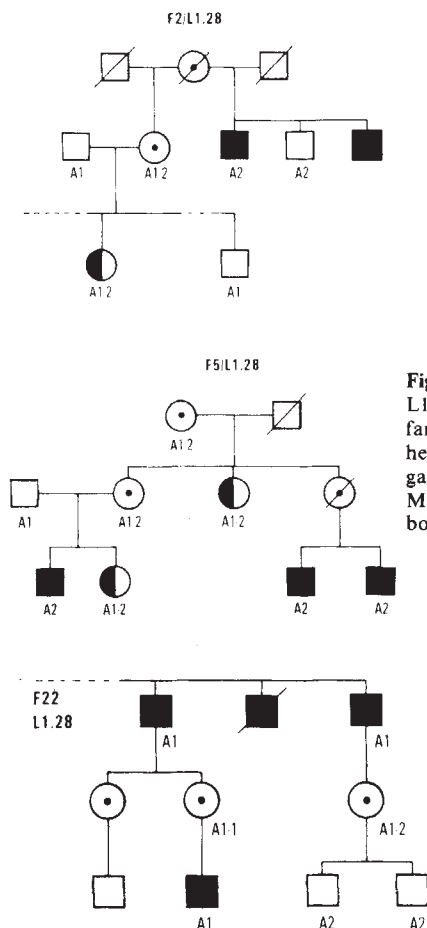


Fig. 2 Pedigrees and L1.28 alleles in XLRP families 2, 5 and 22. XLRP heterozygotes: $\odot$, obligate; $\bullet$, on testing. Methods and other symbols as described in Fig. 1 legend.

firmed by psychophysical and electrophysiological tests, were taken to be XLRP heterozygotes; otherwise the genetic status of females was not inferred unless the pedigree structure showed them to be obligate heterozygotes. Blood samples were obtained from 250 members of 24 XLRP families and lymphoblastoid cell lines were raised from obligate heterozygotes and individuals over 60 yr old.

Estimates of the genetic length of the X chromosome (150–250 centimorgans)¹⁵ suggest that it should be possible to localize the XLRP gene with 10 suitably spaced X chromosome-specific recombinant DNA probes capable of identifying RFLPs. Three such probes assigned to the X chromosome short arm, each of which detects RFLPs with the same restriction endonuclease,

TaqI, have been used so far; one of these, L1.28, was isolated from a human genomic library prepared from the placental DNA of a single individual by Pearson *et al.*¹⁶. The L1.28 probe is a single-copy X chromosome-specific DNA sequence, 1.25 kilobases (kb) long, that has been inserted in the *EcoRI* site of plasmid pBR322. It has been regionally assigned to Xp11.0-p11.3 by using somatic cell hybrids¹⁶. This probe identifies two RFLP alleles with the enzyme *TaqI*: A1, which is 12 kb long and A2, which is 9 kb long. These alleles occur at frequencies of 0.68 and 0.32 respectively in the Dutch population, on the basis of an analysis of 40 chromosomes. Digestion of DNAs from normal individuals using nine other restriction enzymes did not identify RFLP with L1.28 (ref. 16). In addition, 50 X chromosomes from 29 normal individuals have been analysed in Edinburgh and the frequencies of A1 and A2 were found to be 0.74 and 0.26. Overall, this suggests a heterozygote frequency of 0.41 (95% confidence limits 0.33–0.47).

Panels of DNA from XLRP obligate heterozygotes previously digested with *TaqI* were screened for RFLP heterozygosity using probe L1.28. Nine individuals, from five kindreds, were informative for the L1.28 polymorphism, out of a total of 28 obligate heterozygotes screened. The segregation of L1.28 alleles in one XLRP sibship is shown in Fig. 1. Linkage analysis was done using the computer program LIPED¹⁷ and checked by hand.

In four of the kindreds, each with typical XLRP, there were no definite recombinants (Figs 1, 2) and the value of the recombination fraction (θ), at which the likelihood is a maximum, is zero with a LOD score of 3.45 (Table 1). The fifth kindred (F15) was described by Hoare as having X-linked choroidoretinal dystrophy¹⁸. After review and subsequent examination, the consensus of opinion (G. W. Hoare, A.C.B. and B. S. Jay) is that the disorder in this kindred is within the clinical spectrum of XLRP. However, as the possibility of a second locus cannot be excluded, the scores for this kindred are presented both separately and together with the other kindreds (Table 1). Only one female in F15 (III.13), identified as a carrier on testing, was found to be a definite recombinant; the remainder were non-recombinant (Fig. 3). In F15, the maximum likelihood value of θ is 0.04 at a LOD score of 4.65. The linkage data are therefore consistent with the F15 locus being identical with, or close to, that in the other kindreds. When the data from all the kindreds are combined, the maximum likelihood value of θ is 0.03, corresponding to a distance of 3 centimorgans, at a LOD score of 7.89. The 95% confidence limits are 0–15 centimorgans and the odds of linkage at θ values less than 0.3 are 15,000:1 (see Table 1).

Xg blood group data have been analysed in eight kindreds typed by Dr R. Sanger and Dr P. Tippett of the MRC Blood Group Unit, London (Table 1). Close linkage is excluded and

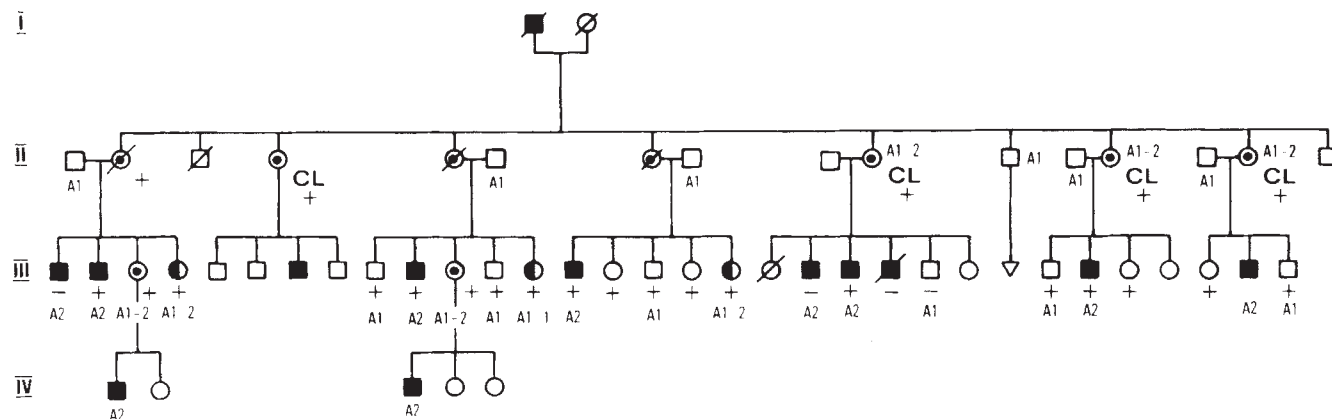


Fig. 3 F15 pedigree with L1.28 and Xg blood group results. This family is highly informative for the L1.28 polymorphism. All of the obligate heterozygotes available in generation II are also heterozygous for the RFLP and, with one exception, all XLRP hemizygotes ($\blacksquare$) and heterozygotes ($\odot$, obligate; $\bullet$, on testing) have inherited allele A2. The exception is III.13, who is homozygous for allele A1 and an XLRP carrier on testing; she is therefore a recombinant. L1.28 results of individuals whose XLRP status is uncertain have not been included in the pedigree. CL denotes that a lymphoblastoid cell line has been raised from that individual. Xg^a(+) and Xg^a(-) alleles are also shown. At least three recombinants between Xg and XLRP can be seen.

the 95% confidence limits for the genetic distance between the XLRP and Xg loci are 38–200 centimorgans. The Xg locus has been assigned to Xp22.3-pter¹⁹ and the genetic length of the short arm of the X chromosome (Xp) is in the region of 60–90 centimorgans¹⁵, so that the assignment of XLRP to within 15 centimorgans of L1.28 (assigned to Xp11.0-p11.3) is consistent with known distances on Xp. Recent evidence from somatic cell hybrids shows that the L1.28 locus is close to Xp11.3 (P.L.P. *et al.*, unpublished), so that while the precise relationship between physical and genetic maps in this region has yet to be established, it is likely that the XLRP locus is on the proximal part of Xp.

The finding of close genetic linkage between XLRP and the L1.28/*TaqI* polymorphism suggests that this could provide a useful means of carrier detection or prenatal diagnosis. However, more XLRP families informative for this RFLP must be examined to exclude significant genetic heterogeneity. Further work is also required to make more families informative for L1.28 and to map XLRP in relation to other neighbouring RFLPs.

S.S.B. was supported by the Sir Jules Thorn Charitable Trust and British Retinitis Pigmentosa Society. We thank Dr S. E. Bunday, S. J. Crews, G. W. Hoare, A. L. Lyness, M. P. Quinlan and A. G. R. Rennie for clinical information, access to patients and providing blood samples; Drs R. Sanger and P. Tappett for

performing the Xg analyses; and Professor A. Laties and the National Retinitis Pigmentosa Foundation for encouragement and support.

Received 23 January; accepted 9 April 1984.

1. Heckenlively, J. in *Principles and Practice of Medical Genetics* Vol. 1 (eds Emery, A. E. H. & Rimoin, D. L.) 522–538 (Churchill Livingstone, Edinburgh, 1983).
2. Jay, B. *Trans. ophthalm. Soc. U.K.* **98**, 309–311 (1978).
3. Boughman, J. A., Conneally, P. M. & Nance, W. E. *Am. J. hum. Genet.* **32**, 223–235 (1980).
4. Hu, D.-N. *Am. J. med. Genet.* **12**, 51–56 (1982).
5. Jay, M. *Br. J. Ophthalm.* **66**, 405–416 (1982).
6. Berson, E. L., Rosner, B. & Simonoff, E. *Am. J. Ophthalm.* **89**, 763–775 (1980).
7. Bird, A. C. *Br. J. Ophthalm.* **59**, 177–199 (1975).
8. Berson, E. L., Rosen, J. B. & Simonoff, E. A. *Am. J. Ophthalm.* **87**, 460–468 (1979).
9. Arden, G. B. *et al. Br. J. Ophthalm.* **67**, 419–430 (1983).
10. Hussels-Maumenee, I., Pierce, E. R., Bias, W. B. & Schleutermann, D. A. *Am. J. hum. Genet.* **27**, 505–508 (1975).
11. Grutzner, P., Sanger, R. & Spivey, B. E. *Humangenetik* **14**, 155–158 (1972).
12. Murray, J. M. *et al. Nature* **300**, 69–71 (1982).
13. Gusella, J. F. *et al. Nature* **306**, 234–238 (1983).
14. Wright, A. F. *et al. Trans. ophthalm. Soc. U.K.* (in the press).
15. Ropers, H.-H., Wieacker, P., Wienker, T. F., Davies, K. & Williamson, R. *Hum. Genet.* **65**, 53–55 (1983).
16. Pearson, P. L., Wieacker, P., Backer, B. & Prins, H. A. *Clin. Genet.* (in the press).
17. Ott, J. *Am. J. hum. Genet.* **26**, 588–597 (1974).
18. Hoare, G. W. *Br. J. Ophthalm.* **49**, 449–457 (1965).
19. Miller, O. J. & Siniscalco, M. *Cytogenet. Cell Genet.* **32**, 179–190 (1982).
20. Clayton, J. F., Bhattacharya, S. S., Wright, A. F. & Price, W. H. *J. med. Genet.* **21**, 141 (1984).
21. Renwick, J. H. *Br. med. Bull.* **25**, 65–73 (1969).
22. Kunkel, L. M., Tantravahi, U., Eisenhard, M. & Latt, S. A. *Nucleic Acids Res.* **10**, 1557–1578 (1982).
23. Southern, E. M. *J. molec. Biol.* **98**, 503–517 (1975).

Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines

Allan Bradley*, Martin Evans*, Matthew H. Kaufman† & Elizabeth Robertson*

* Department of Genetics and † Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK

The recent availability in culture of embryo-derived pluripotent cells which exhibit both a normal karyotype and a high differentiative ability^{1–3} has encouraged us to assess the potential of these cells to form functional germ cells following their incorporation into chimaeric mice. We report here the results of blastocyst injection studies using three independently isolated XY embryo-derived cell lines (EK.CP1, EK.CC1.1 and EKCC1.2) which produce a very high proportion (>50%) of live-born animals that are overtly chimaeric. Seven chimaeric male mice, derived from these three lines, have, so far, proved to be functional germ-line chimaeras.

Although teratocarcinoma-derived embryonal carcinoma (EC) cells have been used to construct chimaeras, they have rarely realized the developmental potential demonstrated by embryonic inner cell mass cells used in a similar combination⁴. Indeed, when introduced into the early embryo many EC cell lines show a low rate of colonization and/or a restricted pattern of differentiation⁵, and many chimaeras develop tumours both pre- and postnatally⁴.

We have recently demonstrated that over 15 of our fertilized and parthenogenetically activated⁶ embryo-derived (EK) cell lines of both XX and XY sex chromosome constitutions form normal chimaeras with high efficiency (in preparation). In this study we decided to use fertilized embryo-derived pluripotent XY cell lines, which, unlike most of their tumour-derived EC cell counterparts, readily differentiate *in vitro* and possess a normal euploid karyotype³, an essential prerequisite for the formation of viable gametes. We now present data which clearly demonstrate that such cell lines are capable of forming functional germ-line chimaeras.

Table 1 gives the results of the blastocyst injection experiments and Table 2 the results of the test breeding studies. Figure 1 shows six of the phenotypically male chimaeras which transmitted functional spermatozoa derived from the introduced cells.

It would obviously be desirable to incorporate specific functional genes into the mouse genome. While direct transformation

Table 1 Rates of construction of chimaeras

Cell line	No. injected	No. born (%)	No. chimaeric (%)	Chimaeras			Males		No. of germ-line chimaeras
				Male	Female	ND	Set up	Bred	
EK.CP1	254	167 (66)	74 (44)	40	27	7	23	20	4
EK.CC1.1	69	52 (75)	31 (60)	21	10	0	13	8	1
EK.CC1.2	160	111 (70)	63 (57)	50	13	0	21	7	2
Total:				111	50			35	7 (20%)

The EK.CP1, EK.CC1.1 and EK.CC1.2 cell lines were isolated from delayed blastocysts¹. All of these lines are characterized by the possession of black and agouti coat colour markers. The EK.CP1 cell line was derived from a 129/Sv/Ev strain mouse and is homozygous for the *GPI-1*^a allele at the *GPI-1* (glucose phosphate isomerase-1) locus. EK.CC1.1 and EK.CC1.2 were derived from a substrain of 129/Sv/Ev which is homozygous for the *GPI-1*^c allele at the *GPI-1* locus. All three of these lines possess a normal euploid XY chromosome constitution and have been maintained entirely *in vitro* on feeder layers of inactivated fibroblasts. The breeding data available for some males are incomplete; these might yet prove to have a low-level culture-derived germ-line component. There is a very evident distortion of the sex ratio in the live-born chimaeras—this deviation in favour of males is highly significant (χ^2 , $P < 0.001$) and probably reflects the conversion of a number of 'host' female embryos to male chimaeras. ND, not determined.

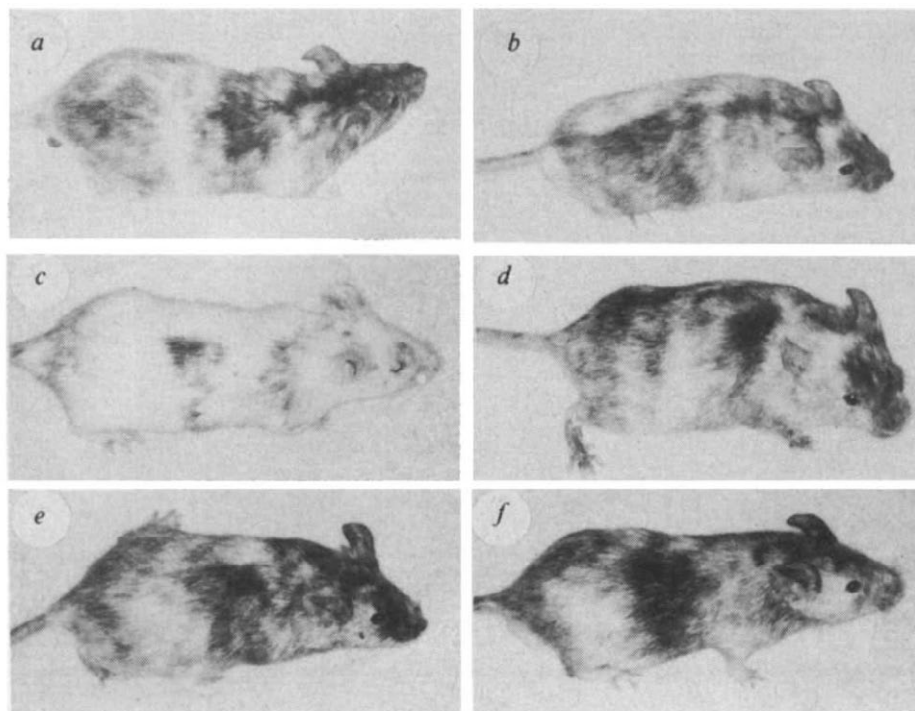


Fig. 1 Six of the seven germ-line chimaeras described in Tables 1 and 2. *a*, CP1.3; *b*, CP1.5; *c*, CP1.11; *d*, CC1.1.3; *e*, CC1.2.6; *f*, CC1.2.8. Between 8 and 12 embryo-derived cells were introduced into the blastocoelic cavity of host-fertilized blastocysts homozygous for the recessive albino locus. The blastocysts were then allowed to re-expand and were subsequently transferred to the uterine lumen of recipients on the third day of pseudopregnancy. All the conceptuses were allowed to develop to term, and live-born animals were scored for the presence of eye and coat pigmentation at or shortly after birth.

of the zygote has recently shown much promise⁷⁻¹³, an alternative approach is the construction of individuals between 'modified' pluripotent cells and normal embryos. This second approach has the advantage that cultured cells are accessible for genetic manipulation, characterization and selection before their incorporation *in vivo*. Several EC cell lines have been selected *in vitro* to carry specific mutations or additional intact 'foreign' chromosomes, and have subsequently been incorporated into chimaeric individuals¹⁴⁻¹⁷. It is, however, a necessary prerequisite to demonstrate that germ-line chimaerism can be reliably achieved. Published rates of construction of germ-line chimaeras using EC cell lines have been disappointingly poor^{18,19}. There has been a report of a single XX cell line, maintained completely *in vitro*, from which two germ-line females have been obtained. In addition to a low rate of chimaera formation (13%), these females produced only four progeny having an EC-derived component^{20,21}.

Clearly, embryo-derived stem cells seem to be particularly efficient at recolonizing the early embryo. This feature, together with the availability of XY lines such as those described here, now allows the routine construction of chimaeric males which are capable of transmitting culture-derived genomes to a potentially limitless number of offspring, and confirms our previous contention of the normality of the genome in these stem cell lines²².

We thank Pam Fletcher for technical assistance. This work was supported by grants from the MRC (M.E. and M.H.K.) and the Cancer Research Campaign (M.E.). A.B. is supported by a MRC Studentship. The EK.CP1 cell line was isolated by Marie-Therese Schnebelen of the College de France, Paris, while in Cambridge on an EMBO short-term fellowship.

Note added in proof: A further four males described in Table 1 have proved to be germ-line chimaeras (three from the CC1.2 and one from the CC1.1 cell lines).

Received 23 January; accepted 20 March 1984.

Table 2 Breeding data from the seven functional germ-line chimaeras

Male chimaera	No. of litters	No. of offspring	No. albino	No. black agouti
CP1.3	10	78	0	78
CP1.5	16	170	163	7
CP1.11	2	23	22	1
CP1.34	1	5	0	5
CC1.1.3	1	12	0	12
CC1.2.6	1	13	0	13
CC1.2.8	1	11	0	11

Phenotypically male chimaeras were caged with successive virgin CFLP females homozygous for the *GPI-1^b* allele and the albino locus. Resulting litters were scored for the dominant black and agouti phenotypes. GPI analysis of blood samples taken from all the black agouti offspring has shown the predicted *GPI-1^a1^b* (EK.CP1) or *GPI-1^b1^c* (EK.CC1.1 and EK.CC1.2) genotype, thus verifying the inheritance of these culture-maintained lines. The chimaeras which appear to be breeding from their XY culture-derived component alone are probably sexual mosaics, although phenotypically they are normal males, as any XX germ cells would not be capable of forming functional spermatozoa.

- Evans, M. J. & Kaufman, M. H. *Nature* **292**, 154-156 (1981).
- Martin, G. R. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7634-7638 (1981).
- Robertson, E. J., Kaufman, M. H., Bradley, A. & Evans, M. J. in *Cold Spring Harb. Conf. Cell Proliferation* **10**, 647-663 (1983).
- Papaioannou, V. E. & Rossant, J. *Cancer Surv.* **2**, 165-183 (1983).
- Papaioannou, V. E., Evans, E. P., Gardner, R. L. & Graham, C. F. *J. Embryol. exp. Morph.* **54**, 277-295 (1979).
- Kaufman, M. H., Robertson, E. J., Handyside, A. H. & Evans, M. J. *J. Embryol. exp. Morph.* **73**, 249-261 (1983).
- Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A. & Ruddle, F. H. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7380-7384 (1980).
- Brinster, R. L. *et al. Cell* **27**, 223-231 (1981).
- Constantini, F. & Lacy, E. *Nature* **294**, 92-94 (1981).
- Wagner, T. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 6376-6380 (1981).
- Jähner, D. & Jaenisch, R. *Nature* **287**, 456-458 (1980).
- Palmiter, R. D. *et al. Nature* **300**, 611-615 (1982).
- Palmiter, R. D., Norstedt, G., Gelinas, R. E., Hammer, R. E. & Brinster, R. L. *Science* **222**, 809 (1983).
- Dewey, M. J., Martin, D. W. Jr, Martin, G. R. & Mintz, B. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5564-5568 (1977).
- Illmensee, K., Hoppe, P. C. & Croce, C. M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1914-1918 (1978).
- Watanabe, T., Dewey, M. J. & Mintz, B. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5113-5117 (1978).
- Illmensee, K. & Croce, C. M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 879-883 (1979).
- Mintz, B. & Illmensee, K. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3585-3589 (1975).
- Illmensee, K. in *Genetic Mosaics and Chimaeras in Mammals* (ed. Russell, L. B.) 3-25 (Plenum, New York, 1978).
- Stewart, T. A. & Mintz, B. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6314-6318 (1981).
- Stewart, T. A. & Mintz, B. *J. exp. Zool.* **224**, 465-496 (1982).
- Evans, M. J. & Kaufman, M. H. *Cancer Surv.* **2**, 185-207 (1983).

Spinal opiates affect sexual behaviour in rats

Z. Wiesenfeld-Hallin* & P. Södersten†

Departments of * Clinical Neurophysiology and † Psychiatry, Karolinska Institutet, S-141 86 Huddinge, Sweden

Enkephalin-containing cells in the substantia gelatinosa of the spinal cord¹ participate in the neural processing of painful stimuli^{2,3}. Spinal opiates may also be involved in the perception of sensory stimuli related to reproduction, as probing of the uterine cervix stimulates sexual behaviour in rats⁴ and also induces an analgesia⁵ which is partially dependent on opiate receptor mechanisms in the spinal cord⁶⁻⁸. We now report that the intrathecal injection of morphine into female ovariectomized rats pretreated with oestradiol benzoate and progesterone inhibits sexual receptivity while injection of the opiate receptor antagonist naloxone enhances it. Similarly, intrathecal injection of morphine increases while injection of naloxone decreases the number of intromissions before ejaculation in male rat. Intraperitoneal injection of morphine or naloxone has no behavioural effect. Recent studies have suggested that brain opiates are involved in the control of sexual behaviour⁹⁻¹¹. The results reported here indicate that sexual behaviour may also be influenced by spinal opiates.

Female Wistar rats (200–220 g) were ovariectomized and 1 week later a polyethylene catheter was inserted into the subarachnoid space of the lumbosacral spinal cord as described elsewhere¹². One week later the rats were injected with oestradiol benzoate and progesterone and tested individually with male rats. All females showed lordosis (concave back flexion, lateral tail deviation and neck extension) to all mounts and were thereafter divided into four groups and injected intrathecally (i.t.) with the opiate receptor agonist morphine in doses which produce moderate analgesia but do not disturb motor function^{13,14}. The number of lordosis responses to 10 mounts by the male was recorded 15 and 60 min after the morphine injection and a lordosis quotient was calculated as: (number of lordosis responses) × 10.

Fourteen ovariectomized rats, pretreated with oestradiol and progesterone, were divided into three groups and injected intraperitoneally (i.p.) or i.t. with morphine. One group given morphine i.t. was injected i.p. with the opiate receptor antagonist naloxone and the other group received an i.p. injection of NaCl. The rats were tested with males 60 min after the morphine injection and lordosis quotients were calculated when the males had mounted 10 times.

Sixteen ovariectomized rats, which showed an overall mean ± s.e.m. lordosis quotient of 43.6 ± 10.5 after pretreatment with a low dose of oestradiol and progesterone were divided into three groups and injected with naloxone i.t., in doses which produce moderate hyperalgesia^{15,16}, or i.p., or with NaCl i.t. The rats were tested with males 10 min after the naloxone injection.

Sexually experienced Wistar male rats (300–350 g) were implanted with i.t. catheters and tested 1 week later for sexual behaviour. A sexually receptive female was introduced to each male and the number of mounts with and without intromission and the latency to the first intromission, the interval between the first intromission and ejaculation and the interval between ejaculation and the next intromission were recorded. Two days later the rats were divided into four groups and injected i.t. with NaCl, naloxone, morphine or morphine + naloxone i.p. and tested for sexual behaviour according to the same time schedule as used with the females. Three other groups of males were injected i.p. with NaCl, naloxone or morphine and tested for sexual behaviour as above.

Figure 1a shows that i.t. injection of morphine suppressed sexual receptivity over time. Between-group comparisons showed that morphine caused a dose-dependent inhibition of lordosis both 15 and 60 min after i.t. injection. As little as 1 µg

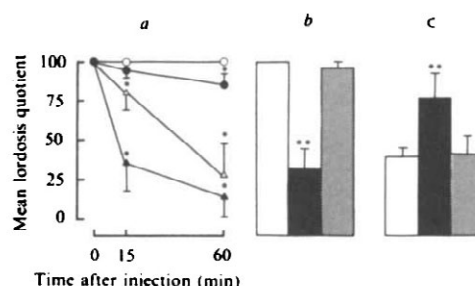


Fig. 1 *a*, Effect of intrathecal (i.t.) injection of 0 (○), 1 (●), 5 (△) or 10 (▲) µg of morphine on sexual receptivity (mean ± s.e.m. lordosis quotient) induced by 10 µg oestradiol benzoate (48 h) and 0.5 mg progesterone (6 h) before the morphine injection. *b*, Effect of intraperitoneal (i.p., open bar) or i.t. (black bar) injection of 5 µg morphine 60 min before testing and of i.t. injection of 5 µg morphine 60 min before testing in combination with 400 µg naloxone i.p. (cross-hatched bar) given 10 min before testing on sexual receptivity induced as in *a*. *c*, Effect of i.p. (open bar) or i.t. (black bar) injection of 50 µg naloxone or 0.9% NaCl i.t. (cross-hatched bar) given 10 min before testing on sexual receptivity induced by 0.8 µg oestradiol (48 h) and 0.5 mg progesterone (6 h) before the naloxone injection. Oestradiol and progesterone (Sigma) were dissolved in 0.1 ml sesame oil and injected subcutaneously. Morphine chloride (Apoteksbolaget) was dissolved in 10 µl 0.9% NaCl and injected i.t. through a Clay Adams PE10 polyethylene catheter or in 0.5 ml NaCl i.p. The i.t. catheter was flushed with 10 µl NaCl after the injection. Naloxone hydrochloride (Endo Laboratories) was dissolved and injected as the morphine. * Significantly different from NaCl-injected controls or ** i.p. morphine-injected (*b*) or i.p. naloxone-injected (*c*) rats; *P* at least <0.05 Mann-Whitney *U*-test after one-way analysis of variance. There were four to six rats per group.

morphine significantly inhibited lordosis behaviour 60 min after injection. Rats given an i.t. injection of morphine showed significantly lower lordosis quotients than rats given morphine i.p., while i.p. injection of naloxone reversed the effect of the i.t. morphine injection (Fig. 1b). Figure 1c shows that an i.t., but not i.p., injection of naloxone facilitated sexual receptivity in rats given a low dose of oestradiol and progesterone.

Males given naloxone i.t. showed fewer and males given morphine i.t. showed more intromissions before ejaculation than males treated with NaCl i.t. (Fig. 2a). The effect of morphine was reversed by an i.p. injection of naloxone (Fig. 2a). None of the other parameters of sexual behaviour was changed by the i.t. injections (data not shown). Intraperitoneal injections of either naloxone or morphine had no effect on intromission frequency (Fig. 2b).

In the female rat, spinal segments L1 to S1 are important for processing sensory stimuli provided by the male during copulation¹⁷. Denervation of peripheral skin¹⁸ or transection of the spinal cord at levels receiving cutaneous information during copulation¹⁹ reduces the intensity of sexual behaviour in female rats. Injection of morphine in the subarachnoid space surrounding these spinal segments produced a dose-dependent inhibition of sexual receptivity in female rats in our study, and this effect was reversed by naloxone. Also, an i.t. naloxone injection facilitated the behaviour of female rats made partially receptive by injection of low doses of ovarian hormones. Since no behavioural effects were noted when either morphine or naloxone was administered at a site distant from the spinal cord, we suggest that one function of dorsal horn opiates in female rats is to modulate sensory stimulation provided by the male during copulation. Thus, stimulation of the uterine cervix of female rats occurs naturally during sexual interactions in the rat²⁰ and it is tempting to speculate that the ensuing analgesia, which is partially opiate dependent², may reflect a copulation-induced release of enkephalins in the dorsal horns of the spinal cord. This, in turn, may underlie such a physiologically relevant phenomenon as copulation-induced inhibition of behavioural oestrus in the female rat²¹.

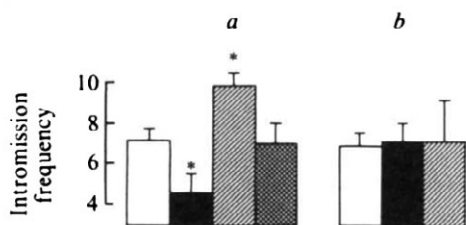


Fig. 2 Effect of intrathecal (a) and intraperitoneal (b) injection of 0.9% NaCl (open bars), 50 µg naloxone (black bars), 5 µg morphine (cross-hatched bars) or 5 µg morphine in combination with 400 µg naloxone i.p. (stippled bar) on intromission frequency (mean $\pm$ s.e.m. number of intromissions before ejaculation) in male rats tested with sexually receptive female rats. Naloxone was given 10 min and morphine was given 60 min before testing. * Significantly different from NaCl-injected controls; $P < 0.05$ Mann-Whitney U -test after one-way analysis of variance. There were four or five rats per group.

During copulation, male rats show multiple intromissions, which serve to facilitate ejaculation²². Anaesthesia of the penis²³ or section of the dorsal penile nerve²⁴ prevents the display of intromission and ejaculation. Thus, sensory stimulation of the penis is required for normal copulation in male rats. It seems likely that spinal opiates may serve a function in coding the sensory information value of each intromission as an i.t. naloxone injection decreased the number of intromissions shown by males before ejaculation and an i.t. morphine injection produced a naloxone-reversible increase in intromission frequency. Naloxone or morphine injections had no behavioural effects if given i.p. Sensory information from the penis is carried by the dorsal penile nerve, the cell bodies of which are located in dorsal root ganglion L6²⁵. These cells have extensive collateral projections to segments T12–S3 in the substantia gelatinosa of the spinal cord²⁵, that is, the segments around which the i.t. injections were made in our study.

Recent work on the physiological role of spinal opiates have concentrated exclusively on their involvement in the perception of pain^{1,2}. The present results suggest that enkephalins in the spinal cord may participate in the coding of a broader range of sensory information.

This work was supported by the Swedish MRC (5318), the Swedish Council for Research in the Humanities and Social Sciences (F224/83) and the Funds of the Karolinska Institute. We thank Apoteksbolaget for supplying morphine and Endo Laboratories for naloxone.

Received 5 December 1983; accepted 19 March 1984.

- Hökfelt, T. et al. in *Brain Stem Control of Spinal Mechanisms* (eds Sjölund, B. & Björklund, A.) 89–117 (Elsevier, Amsterdam, 1982).
- Yoshimura, M. & North, A. *Nature* **305**, 529–530 (1983).
- Yaksh, T. & Hammond, D. L. in *Brain Stem Control of Spinal Mechanisms* (eds Sjölund, B. & Björklund, A.) 437–497 (Elsevier, Amsterdam, 1982).
- Komisaruk, B. R. & Diakow, C. *Endocrinology* **93**, 548–557 (1973).
- Komisaruk, B. R. in *Brain Stem Control of Spinal Mechanisms* (eds Sjölund, B. & Björklund, A.) 493–508 (Elsevier, Amsterdam, 1982).
- Henry, J. L. *Neurosci. Lett.* **38**, 257–262 (1983).
- Hill, R. G. & Ayliff, S. J. *Pharmac. Biochem. Behav.* **14**, 631–632 (1981).
- Crowley, W. R., Rodriguez-Sierra, J. F. & Komisaruk, B. R. *Psychopharmacology* **54**, 223–225 (1977).
- Sirinathsinghji, D. J. S., Whittington, P. E., Audsley, A. & Fraser, H. M. *Nature* **301**, 62–64 (1983).
- Sirinathsinghji, D. J. S., Rees, L. H., Rivier, J. & Wale, V. *Nature* **305**, 232–235 (1983).
- Wiesner, J. B. & Moss, R. L. *Soc. Neurosci. Abstr.* **8**, 930 (1982).
- Yaksh, T. & Rudy, T. A. *Physiol. Behav.* **17**, 1031–1036 (1976).
- Yaksh, T. L. *Pain* **11**, 293–346 (1981).
- Yaksh, T. & Rudy, T. A. *J. Pharmac. exp. Ther.* **202**, 411–428 (1977).
- Dickenson, A. H., Le Bars, D. & Besson, J. M. *Neurosci. Lett.* **24**, 161–164 (1981).
- Wolf, C. J. *Brain Res.* **189**, 593–597 (1980).
- Kow, L. M. & Pfaff, D. W. *J. Neurobiol.* **6**, 1031–1036 (1976).
- Kow, L. M. & Pfaff, D. W. *Brain Res.* **101**, 47–66 (1976).
- Kow, L. M., Montgomery, M. O. & Pfaff, D. W. *Brain Res.* **123**, 75–88 (1977).
- Komisaruk, B. R. in *Biological Determinants of Sexual Behaviour* (ed. Hutchinson, J. B.) 349–393 (Wiley, New York, 1978).
- Lodder, J. & Zeilmaker, G. N. *J. comp. physiol. Psychol.* **90**, 925–929 (1976).
- Larsson, K. in *Endocrine Control of Sexual Behaviour* (ed. Beyer, C.) 77–163 (Raven, New York, 1979).
- Carlsson, S. G. & Larsson, K. *Z. Tierpsychol.* **21**, 854–856 (1964).
- Larsson, K. & Södersten, P. *Physiol. Behav.* **10**, 567–571 (1973).
- Nunez, R., Gross, G. H. & Sachs, B. D. *Soc. Neurosci. Abstr.* **9**, 530 (1983).

A vasopressin-like peptide in the mammalian sympathetic nervous system

Michael R. Hanley*, Hilary P. Benton†, Stafford L. Lightman‡, Kathryn Todd‡, Elisabeth A. Bone§, Pamela Fretten§, Susan Palmer§, Christopher J. Kirk§ & Robert H. Michell§

* Department of Biochemistry, Imperial College of Science and Technology, South Kensington, London SW7 2AZ, UK

† Cancer Research Campaign Cell Proliferation Unit, Department of Histopathology, Royal Postgraduate Medical School, Ducane Road, London W12, UK

‡ Medical Unit, Charing Cross and Westminster Medical School, Page Street Wing, Westminster Hospital, Page Street, London SW1P 2AP, UK

§ Department of Biochemistry, University of Birmingham, PO Box 363, Birmingham B15 2TT, UK

Vasopressin was among the first mammalian hormonal peptides to be identified and to have its structure determined¹. Its only undisputed physiological role is as a circulating neurohypophyseal antidiuretic hormone. Other notable effects of vasopressin on peripheral tissues include contraction of vascular smooth muscle, leading to elevation of blood pressure, and activation of glycogenolysis in liver. It has long been clear that vascular smooth muscle and hepatocytes are relatively insensitive to the low concentrations of vasopressin normally present in the circulation, and the physiological significance of their responses has therefore been in doubt. We now report that a new bioactive and immunoreactive vasopressin-like peptide (VLP) is widely distributed in the sympathetic nervous system of mammals, both in the principal noradrenergic neurones of ganglia and in nerve fibres innervating peripheral tissues. In addition to other peptides described in the mammalian sympathetic nervous system², VLP must be considered as a possible mediator of the non-adrenergic responses to sympathetic activation. Moreover, many of the effects previously attributed to circulating vasopressin may be neurally evoked.

Using several antisera having high specificity for arginine-vasopressin (AVP, Table 1 legend), significant quantities of VLP were detected in rat superior cervical and coeliac ganglia (Table 1). The levels detected in the coeliac ganglia were one-quarter those found in the superior cervical ganglia, suggesting that there may be some rostral-caudal differences in the levels of VLP along the sympathetic chain, as has been found for vasoactive intestinal polypeptide (VIP)³. Detectable levels of oxytocin-like immunoreactivity were also found by using an oxytocin-specific radioimmunoassay, but the levels were about one-tenth those found for VLP (Table 1). The apparent VLP levels in rat superior cervical ganglia are comparable to the levels of substance P-like immunoreactivity expressed after explant culture of the ganglia⁴.

The Brattleboro rat, a mutant of the Long-Evans strain, lacks AVP and consequently proved an important tool in vasopressin research⁵. Specific radioimmunoassays demonstrated that the superior cervical ganglia of Brattleboro rats had significant levels of both vasopressin-like and oxytocin-like immunoreactivity, although the levels of the former were 40% of those measured in Long-Evans controls (Table 1).

Gel filtration established that VLP migrates as a single immunoreactive peak with a higher apparent molecular weight than authentic vasopressin (Fig. 1). Because VLP was extracted from pooled ganglia for gel filtration experiments, it should be considered that the material present at nerve endings may be a processing product of VLP detected in ganglia. The oxytocin-like immunoreactivity co-migrated with VLP, suggesting that the oxytocin and vasopressin antisera recognized a common peptide. Ganglion extracts contained no authentic AVP on analysis by HPLC.

The cross-reactivity of VLP with some, but not all, AVP and oxytocin antisera permitted an investigation of its distribution

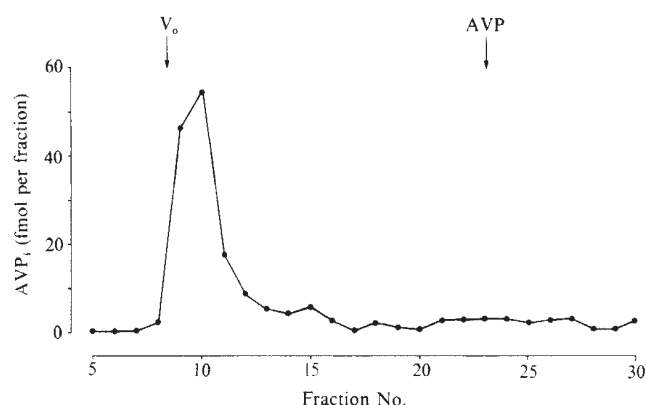


Fig. 1 Elution profile of arginine-vasopressin immunoreactivity from extracts of rat superior cervical ganglia on Sephadex G-25. Extracts were prepared by homogenizing pooled, frozen ganglia from 10 Sprague-Dawley rats in 0.5 ml 50 mM HCl with an ultrasonic disintegrator (MSE Soniprep 150). The homogenate was extracted through acidified octadecylsilica columns (Table 1 legend) and resuspended in 1 ml 0.1 M Tris-HCl buffer (pH 7.4). This extract was applied to a 1.0 × 30 cm Sephadex G-25 (fine) column equilibrated in the same buffer. The column was run in this buffer at 45 ml per h at room temperature and 1-ml samples were collected. Aliquots were assayed for arginine-vasopressin immunoreactivity by the technique described in Table 1 legend. Recovery was 70% of that in the applied sample. The void volume (V_0) and the elution position of authentic arginine-vasopressin (AVP) are indicated by arrows. Aliquots were also assayed for oxytocin immunoreactivity, which co-migrated with the AVP-immunoreactivity (AVP).

by immunocytochemical techniques. Using immunofluorescence, superior cervical, coeliac and thoracic ganglia from two mammalian species (rat, macaque monkey) revealed staining of large neurones in the ganglia (Fig. 2). Because it seemed likely that this staining was restricted to noradrenergic principal neurones, sections of superior cervical ganglia were stained first for VLP and subsequently for dopamine- β -hydroxylase (DBH). All VLP-positive neurones were also stained by anti-DBH, although it is not clear whether all DBH-positive neurones are VLP-positive. It is clear, however, that this pattern is not specific to any particular ganglion or species; it appears to be widespread in the sympathetic nervous system. In some sections, a plexus of nerve fibres could be detected (Fig. 2c) surrounding the large neurones. Because the plexus stains for both VLP and DBH, it seems likely that it arises from postganglionic neurones, although its precise anatomical origin is unknown.

Sympathetically innervated tissues were examined for peripheral fibres that might correspond to the projections of the VLP-positive neurones. In liver and kidney, two known targets of vasopressin, numerous immunoreactive fibres were detected. In the hilar region of the liver, VLP-positive nerve fibres were found running in the smooth muscle layers of the hepatic blood vessels (Fig. 3b), but none was found in apposition to the bile ducts. Similarly, blood vessels of the kidney, particularly the renal artery (Fig. 3a), showed a high density of immunoreactive fibres. In the kidney, fibres were also found in a variety of other structures, for example, in the vicinity of collecting ducts. This is of considerable interest, as vasopressin exerts more than one action on the kidney. A detailed investigation of the renal VLP-positive fibres at both the light and electron microscope level may reveal unexpected possibilities for direct neural control. Similar VLP-positive fibres have been observed in other sympathetically innervated peripheral organs, such as rat submandibular gland.

Because activation of V_1 vasopressin receptors in liver and artery stimulates inositol lipid breakdown⁶, we examined

Table 1 Levels of vasopressin- and oxytocin-like immunoreactivity in rat sympathetic ganglia, and comparison with published concentrations of some other immunoreactive neuropeptides in rat sympathetic ganglia

Immunoreactive peptide	Superior cervical ganglion (fmol per ganglion)		Coeliac ganglion (fmol per ganglion)
	Normal Long-Evans rat	Brattleboro rat	Normal Long-Evans rat
Vasopressin-like	211 ± 26.5* (12)	85 ± 11.5* (12)	58.2 ± 8.8* (8)
Oxytocin-like	23 ± 0.8* (14)	25 ± 1.6* (13)	ND
Substance P-like	Normal Sprague-Dawley rat		Normal Sprague-Dawley rat
	30–40 (ref. 15)		270 ± 30 (ref. 15) 180 ± 40 (ref. 16)
Bombesin-like	ND		3,900 ± 800 (ref. 16)

* Although the recorded concentrations of vasopressin- and oxytocin-like immunoreactivity are based on standards of arginine-vasopressin and oxytocin, the peptide present in ganglia is neither of these (see the text and Fig. 1) and these assays are therefore likely to underestimate the true concentration of ganglionic VLP. The values given are means ± s.e.m. (number of ganglia analysed). ND, not determined. Rats were anaesthetized with sodium pentobarbitone (72 mg per kg) and the superior cervical and coeliac ganglia were dissected and frozen. Each ganglion was then suspended in 0.5 ml 50 mM HCl, homogenized using an ultrasonic homogenizer (MSE Soniprep 150), and centrifuged for 5 min at 11,600g. The supernatant was decanted and neutralized with an equal volume of 0.1 M Tris HCl + 10 μ l 1 M NaOH. A 0.1-ml aliquot of the neutralized homogenate was added to 1 ml 0.1 M HCO_2H , and passed through an acidified octadecylsilica column (C_{18} Sep-Pak; Waters Associates). The column was washed with 3 ml 0.1 M HCO_2H and eluted with 3 ml 95% methanol/5% 0.1 M HCO_2H . The eluate was dried *in vacuo*, resuspended in 0.5 ml assay buffer, and assayed in duplicate by a modification of the technique of Lightman and Forsling¹⁷ at three serial dilutions. In brief, ~4,000 c.p.m. of [¹²⁵I]-arginine-vasopressin tracer (NEN, 1,820 Ci per g) was added and samples were incubated for 48 h at 4 °C. Free and bound tracer were separated by ethanol precipitation, and the bound fraction was counted by a γ -counter. The anti-vasopressin antiserum used here was kindly provided by Stefan Lundin (Ferring Pharmaceuticals). Its cross-reactivity with oxytocin is 0.1%, with lysine-vasopressin 10%, and with desamino-D-arginine vasopressin 0.01%.

inositol lipid metabolism in rat sympathetic ganglia in order to detect functional V_1 receptors. Rat superior cervical ganglia were stimulated with AVP after incubation with [³H]-inositol, and the production of inositol phosphates from inositol lipids was measured⁷. The response was rapid, with detectable accumulation of inositol bis- and trisphosphates within 15 s (Table 2a), suggesting primary breakdown of phosphatidylinositol 4,5-bisphosphate, as in liver⁸. Stimulated inositol lipid breakdown is also a response of these ganglia to muscarinic cholinergic stimulation, but the response to AVP is several times larger⁹. The half-maximal response to AVP occurred at ~30 nM AVP⁹, and was blocked by a specific V_1 -receptor antagonist (Table 2b). The AVP-stimulated accumulation of inositol phosphates was unaffected by a reduction in extracellular Ca^{2+} concentration, suggesting that it is a primary receptor-coupled event rather than a secondary response to another mediator released from AVP-sensitive neurones. Ganglia showed little or no change in cyclic AMP content when exposed to AVP, indicating that they possess few, if any, functional V_2 receptors. The matching of the biochemical responses in ganglia with those in sympathetic target tissues suggests that this is a coordinated system in which VLP may be the preferred endogenous stimulant of V_1 responses.

To test the biological actions of VLP, a sympathetic ganglion extract was freed of catecholamines by adsorption to a miniature reverse-phase column. When injected intravenously into alcohol-anaesthetized, water-loaded rats, the extract had a marked antidiuretic effect with no effect on blood pressure. Thus, although VLP shares at least one biological action with AVP, the relative potencies at V_1 and V_2 receptors cannot yet be assessed.

The simplest interpretation of the data is that VLP is a novel neuropeptide in postganglionic sympathetic neurones that contain and secrete noradrenaline. In this regard, it is important to establish the relationships between VLP and other neuropeptides detected in mammalian sympathetic ganglia. Only neuropeptide Y appears to have a similar distribution to that of VLP¹⁰.

Fig. 2 Immunofluorescence micrographs of vasopressin-like peptide in sympathetic ganglia. *a*, High power view of the edge of a macaque monkey (*Macaca cynomologous*) coeliac ganglion stained for AVP ($\times 154$). Note that the nuclei are unstained. *b*, High power view of rat superior cervical ganglion stained for AVP ($\times 154$). *c*, Rat superior cervical ganglion examined in a focal plane which shows an AVP-positive nerve fibre plexus surrounding the principal neurones ($\times 77$). The principal neurones are unfocused and as a result do not appear clearly stained. *d-f*, A representative double-staining series using the elution method of Tramu *et al.*¹⁸. *d*, View of rat superior cervical ganglion stained for AVP ($\times 98$). *e*, View of the same section after elution of anti-AVP antibody and re-staining with second antiserum (TRITC swine anti-rabbit IgG) to confirm successful elution ($\times 98$). The exposure time for *e* was increased 10-fold to enhance the background for identification of neurones. *f*, View of the same section subsequently stained for the noradrenergic marker, dopamine- β -hydroxylase (DBH) using anti-DBH antiserum. All of the neurones stained in *d* are also stained in *f*. Arrows in each of *d-f* illustrate a representative pair of cells that stained for both VLP and DBH.

Methods: Animals were in all case transcardially perfused with a fixative consisting of 4% freshly prepared *p*-formaldehyde in phosphate-buffered saline (PBS). Tissues were removed and post-fixed for 12–24 h in the same fixative, then sunk in 30% sucrose in PBS overnight at 4 °C. Tissues were rapidly frozen and cut on a cryostat at 10 μ m onto subbed slides. Slide-mounted sections were incubated with primary antibody for 60 min at 37 °C or overnight at 4 °C in 0.2 ml of PBS containing 0.3% Triton X-100 and 3% swine serum. Antisera (AVP, gift of Drs C. Pol and F. Van Leeuwen) were used at a dilution of 1:100. Sections were rinsed with several changes of PBS at room temperature for 30 min, then incubated for 60 min at 37 °C with secondary antiserum (Dako TRITC-labelled swine antiserum against rabbit IgG, diluted 1:50 in PBS/0.3% Triton X-100/3% swine serum). After rinsing in several changes of PBS, sections were examined by fluorescence optics in a Reichert-Jung Polyvar microscope. Controls included staining with secondary antibody (TRITC swine anti-rabbit IgG) alone and pretreatment of antisera with excess antigen. In the latter case, antisera were incubated in the presence of 10 μ g synthetic AVP (CRB Biochemical) or 10 μ g synthetic oxytocin (CRB)—both blocked the specific staining, suggesting that the VLP had antigenic characteristics of both AVP and oxytocin. One oxytocin antiserum (gift of Drs C. Pol and F. Van Leeuwen) produced identical results in immunofluorescence to those obtained with the AVP antibody.

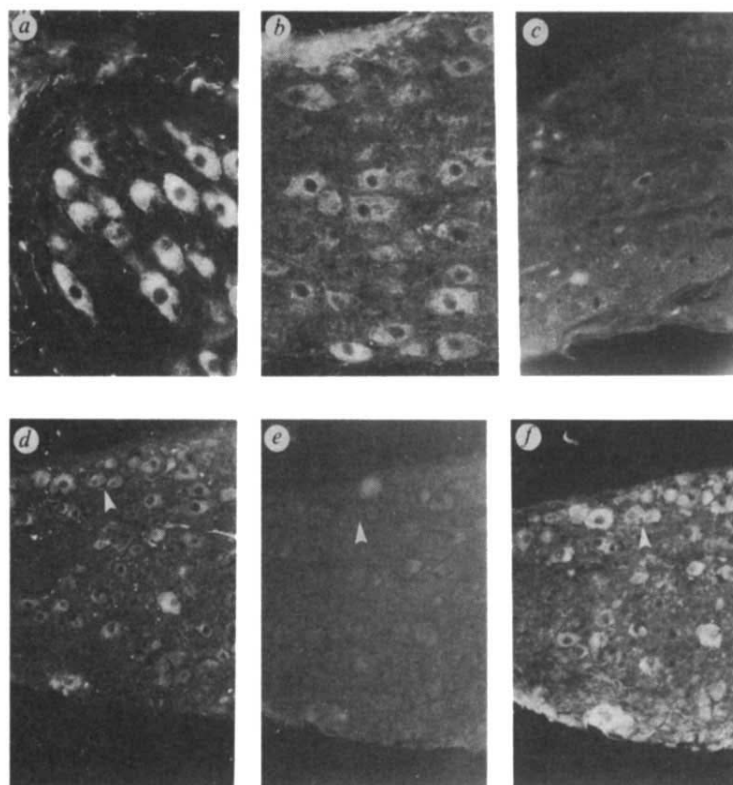


Table 2 Production of inositol phosphates in sympathetic ganglia incubated with AVP

	GroPIns	³ H (d.p.m. per 10 ⁵ d.p.m. in lipids)	InsP ₂	InsP ₃	n
a, Control	499 ± 92	2,209 ± 244	886 ± 142	182 ± 21	15
0.32 μ M AVP for 15 s	496 ± 116	2,173 ± 281	1,325 ± 93†	458 ± 44*	15
b, Control	1,130 ± 150	6,060 ± 650	1,250 ± 140	670 ± 120	14
0.13 μ M AVP for 90 min	1,680 ± 410	160,000 ± 31,000*	29,400 ± 5,850*	3,980 ± 1,220*	9
0.13 μ M AVP and 1 μ M V ₁ antagonist for 90 min	1,150 ± 160	8,740 ± 2,600	1,490 ± 340	450 ± 110	9

a, Superior cervical ganglia from male Wistar rats (~225 g) were desheathed and incubated in 0.3 ml of Krebs–Ringer bicarbonate medium for 2.5 h in the presence of 7.5 μ Ci [2-³H]inositol (16.9 Ci mmol⁻¹; Amersham) at 37 °C. The ganglia were then incubated in inositol-free medium for a further 1 h. Following incubation with Krebs–Ringer medium containing 10 mM LiCl for 5 min (to inhibit breakdown of inositol 1-phosphate⁷) the ganglia were stimulated with 0.32 μ M VP for 15 s. Incubation was terminated with 0.1 ml of 10% (w/v) perchloric acid. The acid-soluble extract was neutralized with 1.53 M KOH (containing 75 mM HEPES) to hold the pH of the neutralized extract at ~7.0. KClO₄ was precipitated at 0 °C and removed by centrifugation. After dilution to 5 ml with 5 mM K⁺ tetraborate and 0.5 mM EDTA, the aqueous extract was applied to a small column of Dowex-1 $\times$ 10 (100–200 mesh, formate form) and inositol phosphates eluted with 15 ml 5 mM sodium tetraborate/60 mM ammonium formate (to elute glycerophosphoinositol, GroPIns), 20 ml 5 mM sodium tetraborate/150 mM ammonium formate (inositol 1-phosphate, InsP), 20 ml 0.1 M formic acid/0.4 M ammonium formate (inositol bisphosphate, InsP₂) and 0.1 M formic acid/1.0 M ammonium formate (inositol trisphosphate, InsP₃). Lipids were extracted from the acid-extracted ganglia using chloroform/methanol/12 M HCl (100:200:1 by vol.). The radioactivity of each phosphate ester was determined, and the values were then adjusted to a standard baseline by calculating the accumulation of radioactivity by reference to a standard incorporation of 10⁵ d.p.m. into the lipids of one ganglion. The results presented are mean $\pm$ s.e.m. of the number of ganglia indicated. Statistical analysis was by an unpaired *t*-test. **b**, Experimental details as in **a**, except that the V₁-vasopressin antagonist ([1-(β -mercapto- β , β -cyclopentamethylenepropionic acid), 2-(*O*-methyl)tyrosine]8-arginine-vasopressin, a gift from Professor M. Manning) was added 5 min before AVP, and incubation was continued for 90 min.

* $P = 0.001$; † $P = 0.02$, difference from appropriate control values.

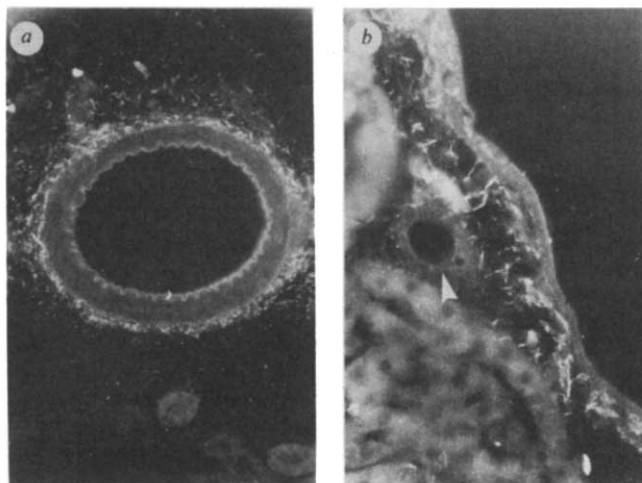


Fig. 3 Immunofluorescence micrographs of vasopressin-like peptide in peripheral nerve fibres of sympathetically innervated tissues. *a*, High power view of cross-section of mouse renal artery stained for AVP ($\times 195$). Note the dense innervation in the outer muscle layers. *b*, High power view of a hepatic blood vessel in the hilar region of rat liver ($\times 195$). Note that the innervation is highly localized to the vascular smooth muscle and is absent from the region surrounding a bile duct (arrow). Tissues were prepared and stained as described in Fig. 2 legend.

Nonetheless, using antisera to avian pancreatic polypeptide, which cross-react with neuropeptide Y¹⁰, we have found that a smaller proportion of DBH-positive cells stain for avian pancreatic polypeptide than for VLP. In addition, the peripheral fibre distribution of immunoreactive pancreatic polypeptide is restricted to vascular beds, where it is less abundant than that of VLP-immunoreactive fibres. The only neuropeptide which has as wide and extensive a distribution of fibres in blood vessels is calcitonin gene-related peptide¹¹, but this is apparently of sensory nerve origin. Significantly, calcitonin gene-related pep-

tide appears to have opposite effects to those of AVP¹², suggesting that peptidergic nerves may provide a dual control of peripheral vasculature. The antisera we used to detect VLP showed negligible reactivity with either neuropeptide Y or calcitonin gene-related peptide.

The peripheral release of VLP may explain why a variety of tissues show responses to AVP only at concentrations higher than are normally achieved by secretion into the bloodstream (see ref. 13). Furthermore, the coexistence of VLP with noradrenaline at sympathetic nerve terminals might provide a simple explanation for some non-adrenergic responses elicited by sympathetic nerve stimulation¹⁴. Such responses, if they are due to VLP, may be conveniently identified by inhibition with selective V₁-receptor antagonists.

M.R.H., E.A.B., P.F., C.J.K. and R.H.M. were supported by MRC grants. S.L.L. was supported by a grant from the Wellcome Trust. H.P.B. and S.P. were supported by MRC studentships. We thank Drs C. Pol, F. Van Leeuwen, J. T. Russell, S. Lundin, I. Robinson and T. Joh for the gift of antisera, Dr I. Robinson for help with HPLC studies, and Professor S. Zeki for providing monkeys.

Received 16 November 1983; accepted 22 March 1984.

1. Du Vigneaud, V. *et al.* *J. Am. chem. Soc.* **25**, 4879–4880 (1953).
2. Lundberg, J. M. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **79**, 1303–1307 (1982).
3. Schultzberg, M., Hokfelt, T. & Lundberg, J. in *Regulatory Peptides of Gut and Brain* (ed. Gregory, R.) 309–313 (Churchill Livingstone, London, 1982).
4. Kessler, J. A., Adler, J. A., Bohn, M. C. & Black, I. B. *Science* **214**, 335–336 (1981).
5. Valtin, H., Schroeder, A., Benirschke, K. & Sokol, H. W. *Nature* **196**, 1109–1110 (1962).
6. Creba, J. A. *et al.* *Biochem. J.* **212**, 733–749 (1983).
7. Berridge, M. J., Downes, C. P. & Hanley, M. R. *Biochem. J.* **206**, 587–595 (1982).
8. Kirk, C. J. *Cell Calcium* **3**, 399–411 (1982).
9. Michell, R. H. *et al.* in *Inositol and Phosphoinositides* (eds Bleasdale, J. E., Eichberg, J. & Hauser, G.) (Humana, New York, in the press).
10. Lundberg, J. M. & Hokfelt, T. *Trends Neurosci.* **6**, 325–333 (1983).
11. Rosenfeld, M. G. *et al.* *Nature* **304**, 129–136 (1983).
12. Fisher, L. A. *et al.* *Nature* **305**, 534–536 (1983).
13. Jard, S. in *Cellular Receptors for Hormones and Neurotransmitters* (eds Schulster, D. & Levitski, A.) 253–266 (Wiley, Chichester, 1980).
14. McGrath, J. C. *J. Physiol., Lond.* **283**, 23–39 (1978).
15. Gamse, R., Wax, A., Zigmond, R. E. & Leeman, S. E. *Neuroscience* **6**, 437–441 (1981).
16. Schultzberg, M. *Neuroscience* **8**, 363–374 (1983).
17. Lightman, S. L. & Forsling, M. *Clin. Endocr.* **12**, 39–46 (1980).
18. Tramu, G., Pillez, A. & Leonardelli, J. *J. Histochem. Cytochem.* **26**, 322–324 (1978).

Voltage-dependent block by Mg²⁺ of NMDA responses in spinal cord neurones

Mark L. Mayer*, Gary L. Westbrook† & Peter B. Guthrie†

* Department of Pharmacology, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK

† Laboratory of Developmental Neurobiology, NICHD, Building 36, Room 2A21, National Institutes of Health, Bethesda, Maryland 20205, USA

Acidic amino acids are putative excitatory synaptic transmitters^{1,2}, the ionic mechanism of which is not well understood. Recent studies with selective agonists and antagonists suggest that neurones of the mammalian central nervous system possess several different receptors for acidic amino acids^{3,4}, which in turn are coupled to separate conductance mechanisms⁵. N-methyl-D-aspartic acid (NMDA) is a selective agonist for one of these receptors^{3,4}. The excitatory action of amino acids acting at NMDA receptors is remarkably sensitive to the membrane potential and it has been suggested that the NMDA receptor is coupled to a voltage-sensitive conductance^{6–9}. Recently, patch-clamp experiments have shown the voltage-dependent block by Mg²⁺ of current flow through ion channels activated by L-glutamate¹⁰. We now show using voltage-clamp experiments on mouse spinal cord neurones that the voltage-sensitivity of NMDA action is greatly reduced on the withdrawal of physio-

logical concentrations (~1 mM) of Mg²⁺ from the extracellular fluid. This provides further evidence that Mg²⁺ blocks inward current flow through ion channels linked to NMDA receptors.

We used spinal cord neurones in dissociated cultures prepared from the ventral half or whole spinal cord of 13–14-day-old mouse embryos as described previously^{11,12}. After 2–4 weeks in culture neurones were impaled with a pair of microelectrodes containing 1 M CsCl, and voltage-clamped¹³. The recording medium contained (in mM): 143 NaCl, 4.8 KCl, 5 CaCl₂, 10 glucose, 10 HEPES and either 0 or 1 mM MgCl₂ and was adjusted to pH 7.3; sucrose was added to match the osmolarity of the growth medium (325 mOsm). Spontaneous activity and synaptic transmission was reduced by adding 0.6 μM tetrodotoxin (TTX). NMDA (1 mM) was dissolved in bathing medium containing 0, 1 or 5 mM MgCl₂ and applied to neurones by pressure using pipettes with tip diameters of 1–2 μm. CdCl₂ (1 or 5 mM) and MgCl₂ (0.5–5 mM, added iso-osmotically to bathing medium) were applied to individual neurones by pressure using pipettes of tip diameter 4–8 μm. Experiments were performed at 28 °C.

Voltage recording showed that in spinal cord neurones bathed in medium containing Mg²⁺ the membrane potential depolarization evoked by NMDA was accompanied by an apparent resistance increase (Fig. 1*a*). This reflects voltage-sensitivity of the response to NMDA, as hyperpolarizing electrotonic potentials used to measure the membrane resistance result in shut off of the voltage-sensitive conductance activated by NMDA^{6–9}. In contrast, when recording in Mg²⁺-free medium, NMDA-evoked depolarizing responses were instead accompanied by a resistance decrease (Fig. 1*b*), as expected if NMDA acts to increase the membrane conductance to ions with a depolarized reversal

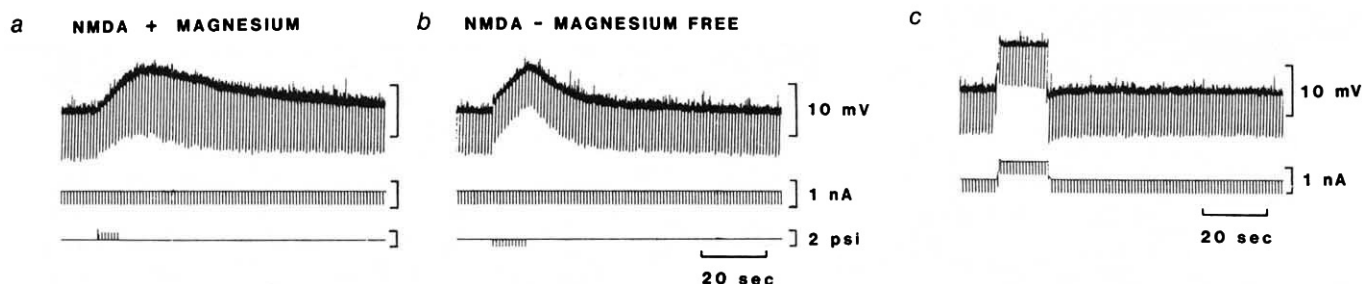


Fig. 1 The apparent membrane resistance change associated with NMDA-evoked depolarizations varies with the extracellular Mg²⁺ concentration. *a*, A depolarizing response evoked by pressure application (110 ms, 1.5 p.s.i., seven pulses) of NMDA (1 mM) dissolved in medium containing 5 mM MgCl₂; note the apparent membrane resistance increase accompanying the NMDA-evoked depolarization. *b*, The response of the same neurone to pressure application (10 ms, 1 p.s.i., 11 pulses) of NMDA dissolved in medium to which no Mg²⁺ had been added; note the resistance decrease accompanying the NMDA-evoked depolarization. *c*, The response to a d.c. current injection, used to depolarize the membrane potential over a similar range to that occurring during the action of NMDA; note the lack of conductance change in contrast to that recorded during the action of NMDA.

Methods: Records of the membrane potential of a mouse spinal cord neurone in dissociated culture bathed in Mg²⁺-free medium, and impaled with a pair of microelectrodes filled with 1 M CsCl. One electrode was used to record membrane potential (upper traces); the second intracellular microelectrode was used to inject hyperpolarizing rectangular current pulses (middle traces: virtual ground current monitor); these evoked electrotonic potentials used to measure membrane resistance. A pressure transducer was used to measure the timing of drug application (lower traces). The actual concentration of NMDA and Mg²⁺ at the membrane surface is unknown, but considerable dilution of the solution ejected from the pressure micropipettes is expected to occur.

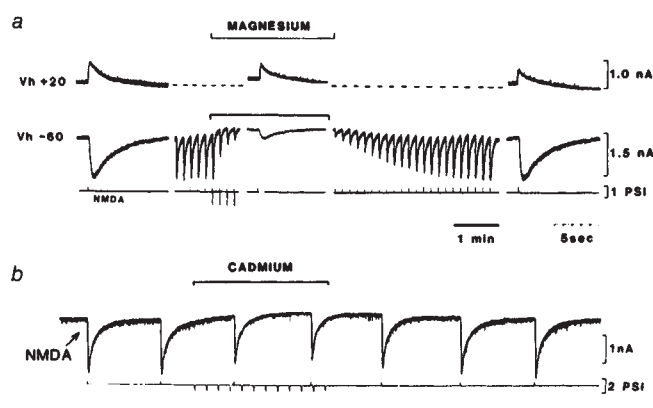


Fig. 2 Mg²⁺ antagonism of NMDA responses is selective and depends on the membrane potential. *a*, Chart records of membrane currents evoked by pressure application (30 ms, 1 p.s.i.) of NMDA (1 mM) in a spinal cord neurone voltage-clamped at holding potentials (V_h) of +20 mV (NMDA currents are outward, that is, upward) and -60 mV (NMDA currents are inward, that is, downward). Pressure application (350 ms, 1.5 p.s.i., five pulses) of Mg²⁺ (5 mM MgCl₂) from a second micropipette reversibly antagonized inward NMDA currents recorded at -60 mV, but had no effect on outward NMDA currents recorded at +20 mV. In Mg²⁺-free medium NMDA currents usually showed slow inactivation, and this is the cause of the small decrease in the amplitude of the NMDA response recorded at +20 mV. *b*, Record obtained for another spinal cord neurone bathed in medium containing 10 mM tetraethylammonium chloride (to suppress outward potassium currents) showing the resistance of NMDA responses (1 mM, 1 p.s.i., 100 ms) to antagonism by doses of Cd²⁺ (1 mM, 1.5 p.s.i., 200 ms, 12 pulses) that completely blocked inward calcium currents evoked by depolarizing voltage jumps (not shown).

Methods: Spinal cord neurones were bathed in Mg²⁺-free medium and impaled with two microelectrodes (1 M CsCl, 60–100 MΩ) for voltage clamping; membrane current was measured with a virtual ground circuit (3 dB, 1.6 kHz; upper traces in *a* and *b*). Pressure transducers were used to measure the period of drug application (lower trace in *a*, middle trace in *b*). In *b* the neurone was voltage-clamped at a holding potential of -50 mV, and 30-ms voltage jumps to -10 mV were used to activate voltage-sensitive inward calcium currents (5 nA peak inward current). Application of Cd²⁺ using the protocol described above blocked the inward Ca²⁺ current such that the depolarizing voltage jump then evoked 2 nA of outward leak current (similar to the inward leak current recorded by a hyperpolarizing voltage jump to -90 mV before and during Cd²⁺ application); despite this complete block of the Ca²⁺ current by Cd²⁺, the NMDA responses recorded in this neurone (*b*) were reduced by only 15%.

potential (for example, Na⁺ or Ca²⁺). To investigate the origin of this effect of Mg²⁺ on the nature of the conductance change evoked by NMDA, it was necessary to perform voltage-clamp experiments.

NMDA evoked inward current (depolarizing) responses in all spinal cord neurones initially voltage-clamped at their resting potential. To study the antagonism by Mg²⁺ of the NMDA response, we recorded from neurones bathed in Mg²⁺-free medium, and applied both NMDA and Mg²⁺ to individual neurones (see Fig. 2*a*). The antagonism of NMDA responses by Mg²⁺ depended closely on the membrane potential: it was strong at -60 mV, but virtually absent at +20 mV. This antagonistic action of Mg²⁺ showed noncompetitive kinetics, that is, in the presence of Mg²⁺ responses evoked by large doses of NMDA were antagonized to a greater extent than responses evoked by lower doses of NMDA. Such results could be explained if Mg²⁺ ions partially entered and blocked channels activated by NMDA, as the binding of Mg²⁺ would be influenced by the membrane electric field. Recent single-channel recording experiments provide clear evidence for a direct action of Mg²⁺ on ion channels activated by L-glutamate¹⁰.

Experiments using intracellular recording from hippocampal pyramidal neurones have been interpreted to suggest that NMDA activates a voltage-sensitive calcium current⁸. However, cadmium (1 and 5 mM), a potent calcium-channel blocker¹⁴, was much less effective as an NMDA antagonist than Mg²⁺ (see Fig. 2*b*). When 1 mM CdCl₂ was added to the recording medium no obvious change in the current-voltage relationship of responses evoked by NMDA was noted. However, in the same neurones for which cadmium had little effect on the response to NMDA, low concentrations of this potent calcium-channel blocker rapidly and completely blocked fast inward calcium currents evoked by depolarizing voltage jumps to -10 or 0 mV. Thus, NMDA does not open the same calcium channels as those gated by membrane potential alone. This result also indicates considerable specificity of the divalent cation-binding site linked to the NMDA-activated channel, with Cd²⁺ having less affinity than Mg²⁺.

The voltage-dependent blocking action of NMDA responses by Mg²⁺ is sufficient to explain the negative slope of the NMDA current-voltage relationship recorded in media containing physiological concentrations of Mg²⁺. Figure 3*a* illustrates the voltage dependence of the NMDA-evoked current recorded in medium containing 1 mM MgCl₂. The current-voltage relationship of the NMDA response has a negative slope over the potential range -70 to -35 mV, indicating that the conductance activated by NMDA becomes smaller on membrane potential

Fig. 3 Voltage-dependence of NMDA responses recorded in spinal cord neurones with and without addition of Mg^{2+} to the extracellular medium. *a, b*, Current-voltage plots of the peak amplitude of NMDA current responses, evoked by pressure application of amino acid to voltage-clamped spinal cord neurones, plotted against the holding potential. Each point is the mean of two responses. *a*, Obtained from a neurone bathed in medium containing 1 mM Mg^{2+} ; note the negative slope of the graph between -35 and -75 mV which indicates voltage sensitivity of the NMDA response. *b*, Obtained from a neurone bathed in medium to which no Mg^{2+} was added; note that the slope is positive at all membrane potentials. The residual voltage sensitivity of the NMDA response recorded in Mg^{2+} -free medium may arise from contamination of the recording medium with trace amounts of Mg^{2+} due to incomplete exchange of the Mg^{2+} -containing growth medium; additional sources of Mg^{2+} contamination may include binding of Mg^{2+} to neuronal membranes, and leakage of Mg^{2+} from the interior of cells. It is also possible that Ca^{2+} has a weak Mg^{2+} -like effect. *c, d*, Chord conductance-voltage plots of the NMDA responses in *a* and *b*, respectively. Note the strong outward rectification of the conductance activated by NMDA in medium containing 1 mM $MgCl_2$: the rectification ratio of the conductance at +20 mV divided by that at -70 mV is 12.2. In Mg^{2+} -free medium the NMDA-activated conductance is considerably less voltage-sensitive, and the rectification ratio recorded over the same membrane potential range is only 2.6.

Methods: Neurones were voltage-clamped as described in Fig. 2 legend and NMDA responses recorded as the membrane potential was changed by 10-mV increments. The chord (ionic) conductance membrane potential relationship was derived as follows: for a parallel conductance model the current flow through an agonist-operated channel (I_x) is related to the conductance of the agonist-operated channel (G_x) by the equation $I_x = G_x(E_m - E_x)$, where E_x is the reversal potential of the current flowing through the agonist-activated channel and E_m is the membrane potential. Thus, we could calculate the relationship between the membrane potential and the NMDA-activated conductance from our measurements of the peak amplitude of the NMDA-evoked current and its reversal potential.

hyperpolarization (see Fig. 3c). In contrast, when $MgCl_2$ was omitted from the recording medium the current-voltage relationship of the response evoked by NMDA did not show the characteristic negative slope behaviour described above. Instead the peak inward current evoked by NMDA increased slightly as the membrane was hyperpolarized over the potential range -40 to -70 mV (see Fig. 1b). Thus, removal of Mg^{2+} from the recording medium greatly reduces the voltage sensitivity of the conductance activated by NMDA (see Fig. 3d).

Previous experiments with NMDA have been interpreted to suggest that this amino acid has a regulatory action on an undefined voltage-sensitive conductance. Initially it was thought that NMDA closed potassium channels^{14,15}. Subsequent studies suggested activation by NMDA of a voltage-sensitive calcium⁸ or sodium^{7,9} conductance. However, our results and those of Nowak *et al.*¹⁰ suggest that physiological concentrations of Mg^{2+} block ion channels linked to NMDA receptors. As the block is voltage-dependent it seems probable that magnesium ions enter the channel and sense the membrane electric field. Channel block by several species of either charged or uncharged molecules is known to occur in a wide variety of membrane ion channels¹⁷⁻¹⁹; the present results extend this situation to ion channels linked to excitatory amino acid receptors. The action of Mg^{2+} at physiological concentrations suggests that acute or chronic changes in the extracellular Mg^{2+} concentration could be potent modulators of neuronal excitability *in vivo*.

We thank Dr Philip Nelson for allowing us to work in his laboratory, Sandy Fitzgerald for preparing the cultures, and Professor J. S. Kelly for his comments on our manuscript. M.L.M. is a Beit Memorial Fellow and thanks the Royal Society for a study visit award to work at NIH.

Received 24 November 1983; accepted 20 February 1984.

- Watkins, J. C. & Evans, R. H. *A. Rev. Pharmac. Tox.* **21**, 165-204 (1981).
- Johnson, J. L. *Prog. Neurobiol.* **10**, 155-202 (1978).
- Watkins, J. C. in *Amino Acid Neurotransmitters* (eds DeFeudis, F. V. & Mandel, P.) 205-212 (Raven, New York, 1981).
- Watkins, J. C., Davies, J., Evans, R. H., Francis, A. A. & Jones, A. in *Glutamate as a Neurotransmitter* (eds DiChiara, G. & Gessa, G. L.) 263-273 (Raven, New York, 1981).
- MacDonald, J. F. & Poriatis, A. V. *Brain Res.* **245**, 175-178 (1982).
- MacDonald, J. F. & Wojtowicz, J. M. *Can. J. Physiol. Pharmac.* **60**, 282-296 (1982).
- MacDonald, J. F., Poriatis, A. V. & Wojtowicz, J. M. *Brain Res.* **237**, 248-253 (1982).
- Dingledine, R. *J. Physiol., Lond.* **343**, 385-405 (1983).
- Flatman, J. A., Schwandt, P. C., Crill, W. E. & Stafstrom, C. E. *Brain Res.* **266**, 169-173 (1983).
- Nowak, L., Bregestovski, P., Ascher, P., Herbet, A. & Prochiantz, A. *Nature* **307**, 492-465 (1984).
- Ransom, B. R., Neale, E., Henkart, M., Bullock, P. N. & Nelson, P. G. *J. Neurophysiol.* **40**, 1132-1150 (1977).
- Guthrie, P. B. & Breneman, D. E. *Soc. Neurosci. Abstr.* **8**, 233 (1982).
- Mayer, M. L. & Westbrook, G. L. *J. Physiol., Lond.* **340**, 19-45 (1983).
- Khrisital, O. A. *Akad. Nauk. SSSR* **231**, 1003-1005 (1976).
- Engberg, I., Flatman, J. A. & Lambert, J. D. C. *Br. J. Pharmac.* **64**, 384-385 P.
- Engberg, I., Flatman, J. A. & Lambert, J. D. C. *J. Physiol., Lond.* **288**, 227-261 (1979).
- Coronado, R. & Miller, C. *Nature* **280**, 807-810 (1979).
- Neher, E. & Steinbach, J. H. *J. Physiol., Lond.* **277**, 153-176 (1978).
- Ogden, D. C., Siegelbaum, S. A. & Colquhoun, D. *Nature* **289**, 596-598 (1981).

Spectral sensitivity of single cones in the retina of *Macaca fascicularis*

B. J. Nunn, J. L. Schnapf & D. A. Baylor

Department of Neurobiology, Stanford Medical School, Stanford, California 94305, USA

Colour vision depends on the wavelength-dependent absorptions of three different photolabile pigments each located in a particular type of retinal cone. The spectral absorption of primate cones has been examined by microspectrophotometry¹⁻⁷, but this method gives information only at wavelengths where the absorption is relatively strong. Here we have analysed the absorption of two of the cones over a wider range of wavelengths by recording their electrical responses to monochromatic light. The observations were made on the retina of the monkey *Macaca fascicularis*, an animal thought to have cone pigments like those of man⁸. The measured spectral sensitivities of the red-sensitive ('red') and green-sensitive ('green') cones agreed well with estimates⁹ of the cone pigment absorptions derived from colour-matching experiments in humans. At long wavelengths the sensitivity of the red cones was found to decline more rapidly than that of the green. This behaviour, attributable to the cone pigment molecules themselves, explains the paradoxical hue shift¹⁰, in which a light of very long wavelength is perceived to be identical to a light of shorter wavelength.

A glass suction electrode was used to record photocurrent from a single cone outer segment projecting from a chopped piece of retina^{11,12}. The recording chamber was continuously perfused with bicarbonate-buffered Locke's solution equilibrated with 95% O₂, 5% CO₂, and the fluid in the chamber kept near 37 °C. Light stimuli were made 'monochromatic' by interference filters of half bandwidth 10 nm. Flash strength was varied by a calibrated series of neutral density filters, and the unattenuated flux density at each wavelength was determined at the end of each day. Spectral sensitivity (the reciprocal of the flash photon density required to evoke a response of constant amplitude) was determined by the method of Naka and Rushton¹³. To prevent errors resulting from progressive bleaching or movement of the cell in the electrode, the sensitivity at each wavelength was measured between runs at 500 nm and expressed relative to the average value at 500 nm. Roughly 1,000 flashes were delivered while determining the entire spectral sensitivity over a period of about 1 h. The light was applied to the outer segment transverse to its long axis. Because of the short path length for transverse incidence, self-screening of the pigment can be neglected and measured sensitivity should be proportional to the molecular extinction coefficient of the pigment in the cone.

Outward-going photocurrents up to 20 pA in saturating amplitude were elicited by brief flashes. Figure 1a shows a family of flash responses from a red cone and Fig. 1b a similar family from a green cone. Flash strengths were increased by factors of about two. The photocurrents were diphasic, with the recovery

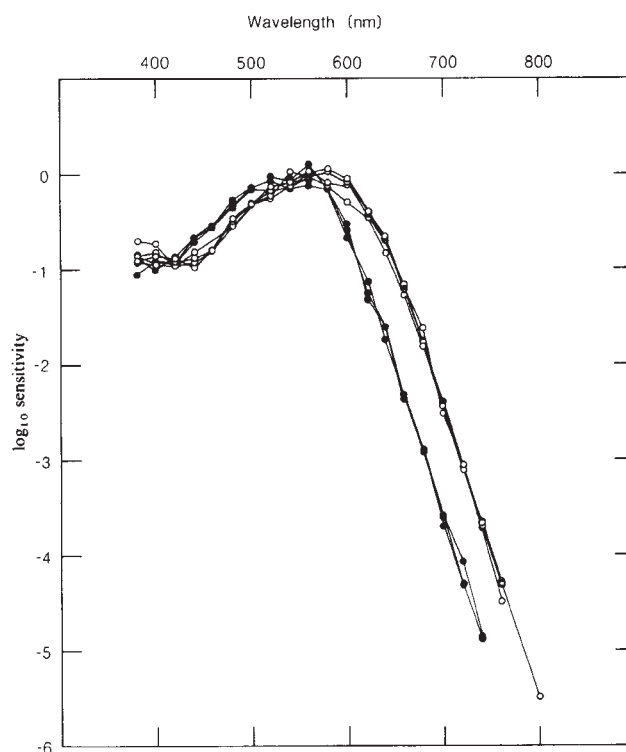
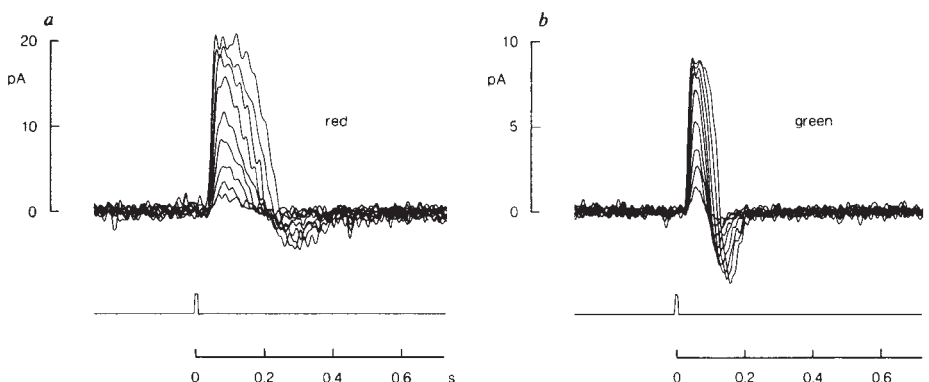


Fig. 2 Collected spectral sensitivities from seven cones. Open symbols, red cones, closed symbols, green. Lines drawn to connect each set of points. Curves positioned vertically to minimize the difference between curves in each population and to give a maximum near $\log S = 0$. Sensitivity determined as described in text. Results from two male monkeys. One red cone was from the foveal region, all others from the peripheral retina.

phase marked by an underswing. The time to peak of the average response to a dim flash was 60–100 ms in six of the seven cells studied, while in one unusually slow green cone it was 150 ms. The small sample of cells provided no evidence for a systematic difference between the kinetics of the responses of red and green cones. The cone responses were, however, two to three times faster than those recorded from single rods in similar preparations from this species¹². As expected from the principle of univariance¹³, the waveforms of a cone's responses to any two monochromatic lights were identical when the light intensities were adjusted to give responses of equal amplitude.

Spectral sensitivity curves from seven cells are plotted in Fig. 2. There was no pre-selection of data; a cell was studied if it gave large responses and did not have the physiological characteristics of a rod¹². The results fell into two groups with spectral maxima at roughly 540 nm (green cones) and 570 nm (red). Microspectrophotometry has given values near 535 and 567 nm for the average peak absorption of these two types of cones in *Macaca fascicularis*^{4,5} and similar values (531, 558 nm) in human

Fig. 1 Superimposed responses to brief flashes of increasing strength from a red cone (a) and a green cone (b) of the peripheral retina. Outward change in membrane current plotted as a function of time after the flash. Light monitor trace below. Each response averaged from up to 10 sweeps. Flash strengths were increased by nominal factors of 2. In a, flashes were at 579 nm and the intensities were raised from 292 to 40,210 photons μm^{-2} ; in b, 559 nm and 453 to 65,640 photons μm^{-2} . Bandwidth, 0–50 Hz. Temperature, 37 °C.



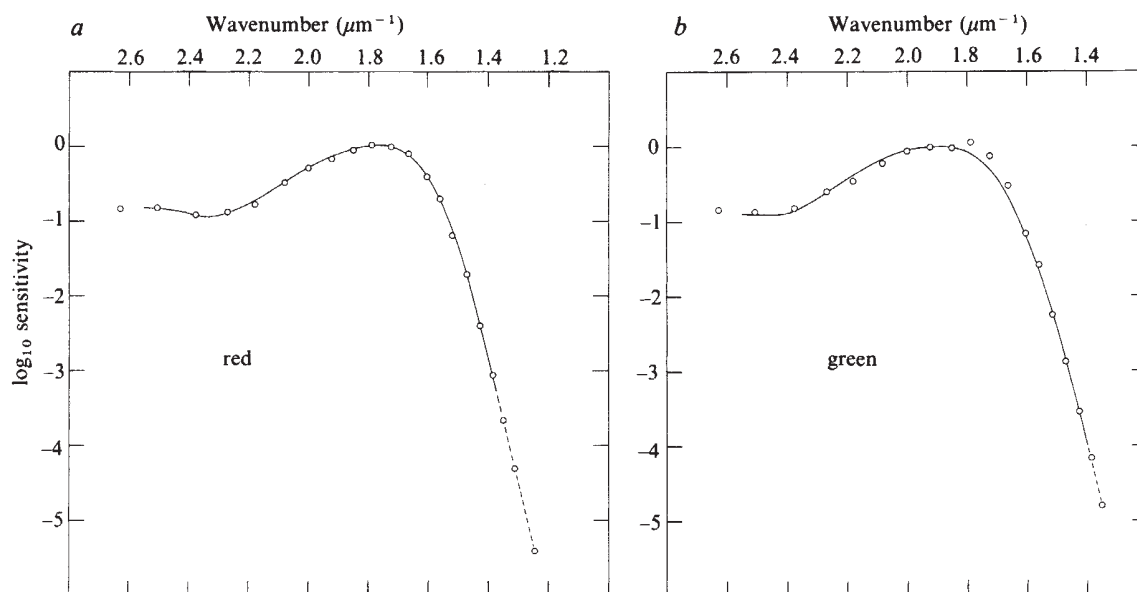


Fig. 3 Averages of $\log_{10}$ spectral sensitivities from the four red and three green cones of Fig. 2, plotted on a wavenumber scale. Solid curves drawn according to estimates by Estevez⁹ of the relative spectral absorption of red and green cone pigments in humans. Each set of points positioned on the vertical scale to best fit the curves, which have maxima at $1.79 \mu\text{m}^{-1}$ (560 nm) in *a* and at $1.85 \mu\text{m}^{-1}$ (540 nm) in *b*.

cones⁷. Blue cones have not been encountered in the present work. From curves of averaged sensitivity, the spectral bandwidths at half height were estimated as $0.37 \pm 0.2 \mu\text{m}^{-1}$ (red) and $0.41 \pm 0.2 \mu\text{m}^{-1}$ (green); these values are comparable to those obtained by microspectrophotometry on cones of the same species⁵.

The averaged spectral sensitivities of the red and green cones are plotted on a wavenumber scale in Fig. 3. The continuous curves show derived spectral absorptions in the red and green pigments as obtained by Estevez⁹ from human colour-matching experiments; the dashed extensions of the curves at low wavenumber have been drawn by eye. Estimates by Smith and Pokorny¹⁴ were similar but did not fit our measurements as well at short wavelength. The psychophysical estimates were calculated on the König assumption¹⁵ that dichromats simply lack one of the three pigments present in normal trichromats. The agreement between the derived and measured sensitivities supports this assumption and further emphasizes the similarity of the primary colour mechanisms in the cynomolgus monkey and man.

The gradient of descent of log sensitivity at long wavelength was steeper for the red cones than for the green as predicted on theoretical grounds by Lewis¹⁶ in 1955. Figure 4 shows this in a wavenumber plot. The lines were obtained by linear regression of $\log_{10}$ sensitivity on wavenumber, using the points at the four lowest wavenumbers. For the red cones, regression analysis gave a gradient of $17.7 \pm 0.4 \mu\text{m}$ (slope $\pm$ standard error; correlation coefficient $r^2 = 0.995$), while for the green it was $15.5 \pm 0.6 \mu\text{m}$ ($r^2 = 0.986$). These gradients are less than the value of $20.0 \mu\text{m}$ derived by Stiles¹⁷ on the assumption that the ground state vibrational energies of the pigment molecules are Boltzmann-distributed. Within experimental error the gradient of decline of the red sensitivity at long wavelength is the same as the gradient of human foveal sensitivity ($17.4 \mu\text{m}$)^{18,19}, as expected if at threshold only red cones mediate foveal sensation in this region of the spectrum. Because the sensitivity gradient of green cones at long wavelength was less steep, the ratio of red/green sensitivities was maximum at roughly 700 nm and less on either side. This explains the psychophysical observation that a monochromatic light beyond 700 nm has the same hue as that at a characteristic wavelength below 700 nm^{10,20}. Estimates of the wavelength pairs that should give the same ratio of excitation in the red and green cones were determined from

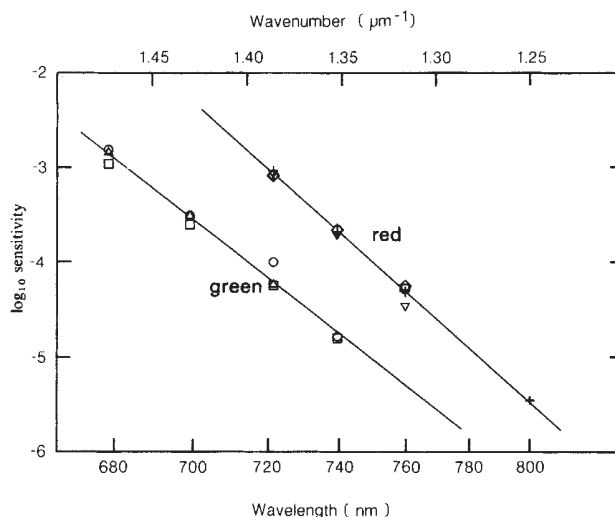


Fig. 4 Decline of sensitivity at low wavenumber. Long wavelength points from Fig. 2 replotted on a wavenumber scale, with results from each cell denoted by a different symbol. Lines obtained from the linear regression analysis described in the text.

the present results and found to be similar to the psychophysical isochromes. This suggests that the perceptual phenomenon may result in a simple way from the characteristics of absorption in the cone pigment molecules.

We thank Mr Mark Siegel and Drs Carolyn Reed, Marla Luskin, Helga Kolb, Walter Makous and Carla Shatz for valuable assistance. This work was supported by US PHS grant EY01543.

Note added in proof: O. Estevez has informed us that the protanopic confusion loci on which his calculations were based are printed incorrectly in ref. 15 (p. 616). The values should read $r_{pc} = 1.0381$ and $g_{pc} = -0.0388$.

Received 1 December, 1983; accepted 14 February 1984.

1. Brown, P. K. & Wald, G. *Science* **144**, 45–52 (1964).
2. Marks, W. B., Dobelle, W. H. & MacNichol, E. F. Jr *Science* **143**, 1181–1183 (1964).
3. Bowmaker, J. K. & Dartnall, H. J. A. *J. Physiol., Lond.* **298**, 501–511 (1980).
4. Bowmaker, J. K. & Dartnall, H. J. A. & Mollon, J. D. *J. Physiol., Lond.* **298**, 131–143 (1980).

5. MacNichol, E. F. *et al.* in *Colour Vision* (eds Mollon, J. D. & Sharpe, L. T.) 14–38 (Academic, New York, 1983).
6. Harosi, F. I. *Color* 7, 135–141 (1982).
7. Dartnall, H. J. A., Bowmaker, J. K. & Mollon, J. D. *Proc. R. Soc. B* **220**, 115–130 (1983).
8. De Valois, R. L. *et al.* *Vision Res.* **14**, 53–67 (1974).
9. Estevez, O. in *Colour Science* (eds Wyszecki, G. & Stiles, W. S.) 620 (Wiley, New York, 1982).
10. Brindley, G. S. *J. Physiol., Lond.* **130**, 35–44 (1955).
11. Baylor, D. A., Lamb, T. D. & Yau, K.-W. *J. Physiol., Lond.* **288**, 589–611 (1979).
12. Nunn, B. J. & Baylor, D. A. *Nature* **299**, 726–728 (1982).
13. Naka, K. I. & Rushton, W. A. H. *J. Physiol., Lond.* **185**, 536–555 (1966).
14. Smith, V. C. & Pokorny, J. *Vision Res.* **15**, 161–171 (1975).
15. Wyszecki, G. & Stiles, W. S. (eds) *Colour Science* (Wiley, New York, 1982).
16. Lewis, P. R. *J. Physiol., Lond.* **130**, 45–52 (1955).
17. Stiles, W. S. *Transactions of the Optical Convention of the Worshipful Company of Spectacle Makers* 97–107 (1948). Reprinted in W. S. Stiles *Mechanisms of Colour Vision* (Academic, New York, 1978).
18. Goodeve, C. F. *Proc. R. Soc. A* **155**, 664–683 (1936).
19. Griffin, D. R., Hubbard, R. & Wald, G. *J. opt. Soc. Am.* **37**, 546–554 (1947).
20. Stiles, W. S. & Burch, J. M. *Optica Acta* **6**, 1–26 (1959).

Localized Ca^{2+} and calcium-activated potassium conductances in terminals of a barnacle photoreceptor

N. Stockbridge & W. N. Ross

Department of Physiology, New York Medical College, Valhalla, New York 10595, USA
The Marine Biological Laboratory, Woods Hole, Massachusetts 02543, USA

Calcium channels are found in the presynaptic terminals of neurones, where they have a key role in synaptic transmission¹. They are also found in the somata of many cells², in dendrites³ and along a few axons^{2,4}. In no cell is the actual distribution of these channels known in detail, because there are no known toxins or other agents suitable for labelling calcium channels, and the current through these channels is usually too small to be quantified with extracellular electrodes. However, several experiments have suggested that the density of the channels is less in the axon than in the cell body or terminal region^{5–8}. Here we have used the indicator dye Arsenazo III in conjunction with an array of photodetectors to examine the spatial influx of calcium in the presynaptic terminal region of the giant barnacle, *Balanus nubilus*. In these cells, calcium entry occurs in a restricted region less than 50 μm in length, which corresponds closely to the region of synaptic contact with second-order cells. Outside this area the magnitude of calcium entry is reduced at least 50-fold. With reasonable assumptions it follows that the calcium channel density is equally localized. In addition, we demonstrate that these cells have a calcium-activated potassium conductance. Since calcium entry is restricted to the synaptic zone, this conductance must be effective only in this region.

Optical experiments using the calcium indicator dye Arsenazo III can show changes in cytoplasmic calcium concentration due to opening of calcium channels⁹. Because calcium ion diffusion within the cytoplasm is slow ($D \approx 3 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$)¹⁰ the channels must be located close to the site of calcium concentration increase. We have used an array of photodetectors^{11,12} with a spatial resolution of 25 μm in the plane of the preparation to measure simultaneously the magnitude and time-course of calcium transients at many positions. Previous experiments have used a single detector and only one looked for the extent of calcium entry: Miledi and Parker¹³, recording successively from two positions in the squid presynaptic terminal with a resolution of 500 μm , showed that calcium entry was greater at the synapse than at a position away from this region.

The photoreceptors of the giant barnacle are well suited for a study of calcium channel localization. From the ocellus each of the four photoreceptors sends a 10–15 mm long axon to the supraoesophageal ganglion which then bifurcates, ending in two restricted synaptic regions on the medial side of each hemi-ganglion (Fig. 1). The dimensions of this area are typical of many synaptic regions in both vertebrates and invertebrates and are

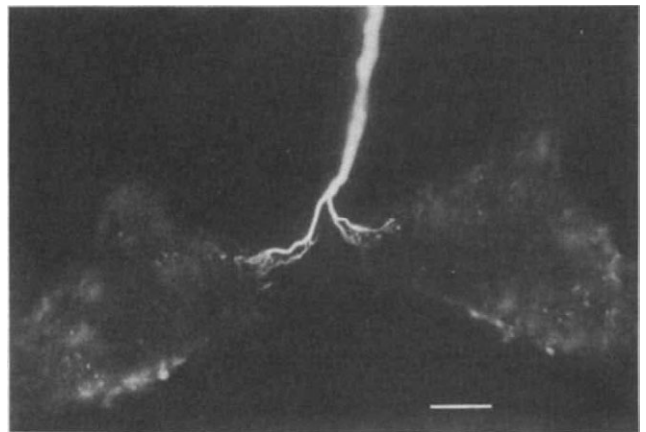


Fig. 1 Photoreceptor axon and terminal region filled with Lucifer yellow and photographed with fluorescence optics. The preparation has been fixed and cleared. The dimmer masses on each side of the terminals are the two hemi-ganglia of the supraoesophageal ganglion. The microelectrode for injecting Arsenazo III and Lucifer yellow was inserted in the axon at a point near the top of the figure. The scale bar represents 150 μm , which is also the diameter of the field sensed by the 32 elements of the photodiode array.

much smaller than the squid giant synapse. The axon can be impaled along its length but the synaptic zones are too small for intracellular recording¹⁴.

In normal physiological saline (461 mM NaCl, 8 mM KCl, 20 mM CaCl_2 , 12 mM MgCl_2 , 5 mM HEPES, pH 7.6) the receptor potential is conducted decrementally to the synapse. In saline containing ≥ 5 mM tetraethylammonium (TEA), a depolarizing potential will generate a calcium-dependent action potential followed by an after-hyperpolarization lasting several seconds⁷. In experiments with two microelectrodes in the axon, the amplitude of both the action potential and the undershoot were found to be larger when recorded from the electrode closer to the terminal, suggesting that the calcium channels might be more concentrated in the terminal region⁷. Pharmacological experiments gave further evidence that there were more channels in the terminal but did not improve the spatial resolution⁸.

For our experiments the photoreceptor axon was impaled near the bifurcation with an electrode containing 75 mM Arsenazo III (Sigma, 8891). The dye was iontophoresed into the axon and allowed to diffuse for 15 min into the fine presynaptic terminals in each hemi-ganglion. The terminal region was imaged onto a square array of 32 photodiodes. In response to a depolarization produced by a brief light flash or electrically via the microelectrode, an absorbance increase at 660 nm was detected by photodiodes positioned over each terminal. The absorbance changes had a biphasic action spectrum consistent with an increase in ionized calcium interacting with the dye¹⁵. In other experiments the absorbance changes were completely eliminated when the 20 mM Ca^{2+} in the saline was replaced with 2 mM Ca^{2+} + 18 mM Co^{2+} or when 30 mM CoCl_2 was added to the saline. The same solutions also block the calcium-dependent action potential which can be elicited when TEA is added to the saline. These observations suggest that the absorbance changes are caused by calcium entering through membrane channels which have a pharmacology typical of other voltage-sensitive calcium channels¹⁶.

Figure 2 shows the change in absorbance at 660 nm detected by each of 32 elements of the photodiode array. The signals have been placed over the outline of a fluorescence photograph of the terminal which was filled with Lucifer yellow after the Arsenazo experiment was completed. The largest signals are located over the final 50 μm of the terminal and are resolved with a signal-to-noise ratio of greater than 50:1. The magnitude declined by a factor of 10 within 50 μm of the terminal and no signal was detected above noise level when the array was moved to cover the axon region at the point of bifurcation in the

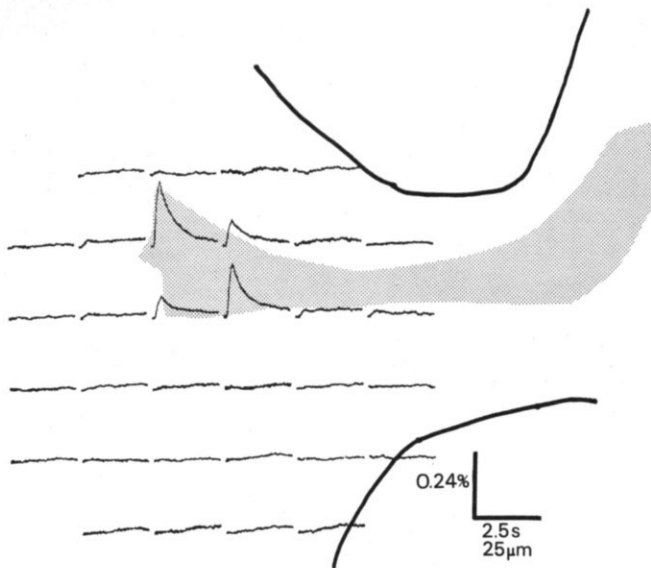


Fig. 2 Spatial distribution of Arsenazo III absorbance changes in the photoreceptor presynaptic terminal region. The stippled area traces the outline of the presynaptic terminal determined from a photograph of the live Lucifer yellow-filled photoreceptor. The heavy lines are the boundary of the ganglion. Each trace shows the stimulus-induced change in light intensity detected by a photodiode element. Fifty sweeps were averaged. The change in absorbance ΔA is proportional to $\Delta I/I$ as long as the dye does not absorb a significant fraction of the incident light, as was true for these experiments. The photoreceptor was stimulated with 250-ms depolarizing pulses through the same microelectrode used to iontophorese dye into the cell. Note that the absorbance increases during the stimulus pulse and declines to baseline with a time constant of about 1.5 s, more than five times faster than that measured at the presynaptic terminal of the squid giant synapse²². **Methods:** To make these measurements, the ganglion was imaged onto the photodiode array using a $\times 40$ water-immersion lens (n.a. = 0.75). The incident wavelength was selected with interference filters having a bandwidth of 30 nm. The current output of each photodiode was converted to voltage and the d.c. component of this signal was subtracted using a sample-and-hold circuit. This signal was RC filtered with a time constant of 10 ms, amplified ($\times 2,000$), multiplexed and digitized into an LSI-11/23 computer for further analysis and display.

commissure. The smaller signals around the central region are probably the result of light scattering, as the signals to the top and left of the terminal come from areas where there is no terminal membrane and hence no dye. Tests with a 20- μm diameter pinhole placed under the ganglion showed that the tissue scattered the transmitted light with almost the same distribution as the absorbance signal. In addition, all of the signals have the same time course, consistent with their originating from a single locus.

In five of six experiments made using the 25- μm spacing of the grid elements, we found that the calcium signals were similarly localized to a 25–50 μm region at the end of the presynaptic process. This corresponds closely to the region of contact with the second-order cells determined anatomically¹⁷. In one experiment the zone of calcium entry extended over 100 μm . This may indicate that some calcium channels exist outside the synaptic zone. A more likely explanation is that the synaptic zone itself is larger than 50 μm in some preparations. The presynaptic arborization shown in Fig. 1 is an example of a cell with a larger than average arborisation. It is unlikely that the absence of signals over the region away from the terminal is due to unusually high buffering of calcium because direct iontophoretic injection of calcium into axons previously filled with Arsenazo III gave large signals.

How does the channel density in the terminal compare with that in the rest of the axon? If we assume that the concentration of Arsenazo III is uniform in the terminal region (reasonable

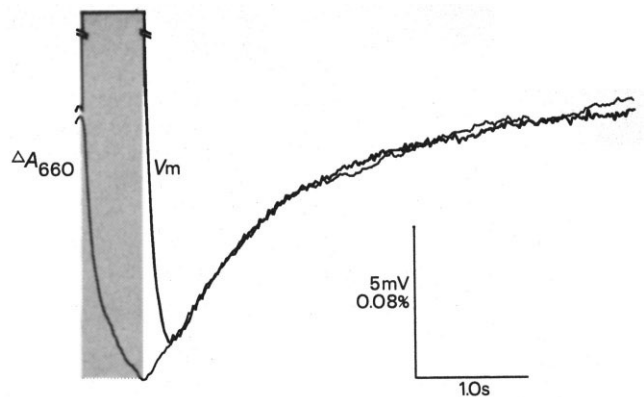


Fig. 3 Comparison of the time course of the undershoot following a depolarizing pulse (stippled area) with the simultaneously measured change in absorbance of Arsenazo III. The signal from one of the photodiodes located over the terminal region has been inverted and scaled to make the two curves coincide at the time when the potential undershoot is largest. The peak of the calcium signal coincides with the end of the depolarizing pulse. However, the peak of the undershoot is not detected at the electrode until about 100 ms later because of the time and space constants of the cell. The magnitude of the depolarizing pulse was not determined; bridge stimulation was used. Ten sweeps were averaged.

since the dye is injected several hundred micrometres from the terminal) and that the absorbance change is proportional to the amount of calcium entry (as shown directly by Miledi and Parker at the squid synapse¹³), it follows that the intensity change detected by each photodiode element is proportional to the amount of calcium entering the cell in the image plane of each element. Since the calcium enters through voltage-sensitive channels and the terminal is uniformly depolarized by the electrode, the signal size is proportional to the number of calcium channels. This proportionality can be extended to channel density if the amount of membrane is the same under each grid element. For this synapse, an examination of photographs of cells filled with Lucifer yellow indicates that there is probably less membrane area per grid element at the synapse than over the axon. Making the conservative assumption of equality, we conclude that the density of calcium channels in the terminal is at least 50 times higher than in the axon. This restricted distribution of calcium channels is reminiscent of the localization of acetylcholine receptor channels on the postsynaptic membrane of the neuromuscular junction¹⁸.

The undershoot following a calcium-dependent action potential elicited in TEA saline has a reversal potential dependent on external potassium concentration and a magnitude dependent on external calcium concentration¹⁹, suggesting that it is caused by a calcium-activated potassium conductance. Further evidence for this conclusion comes from a comparison between the Arsenazo III signal and the undershoot elicited with a depolarizing pulse in normal saline (Fig. 3). After the first 100 ms following the depolarizing pulse the potential returns to baseline with the same time course as the reduction in intracellular calcium. Because the voltage is always far from the potassium equilibrium potential, the conductance change also follows the time course of the intracellular calcium concentration change. This correspondence is expected if the average calcium concentration in the terminal parallels the concentration directly under the membrane where the conductance is controlled²⁰. We have modelled the diffusion of calcium within volumes typical of the photoreceptor terminal using the methods of Zucker and Stockbridge²¹. The time to reach 90% of the equilibrium after a pulse of calcium enters the terminal was found to be $t \approx kBr^2$ where $k = 4 \times 10^{-4}$, B is the ratio of the diffusion constant of calcium in water to that in cytoplasm, and r is the radius of the synaptic terminal assumed to be a cylinder. If $B = 20$ (ref. 10)

$r = 4 \mu\text{m}$ (ref. 17), then $t = 128 \text{ ms}$, which is fast compared with the observed time course of calcium removal.

Since calcium entry is restricted to the terminal, the calcium-activated conductance change is similarly restricted. The potassium channels themselves may have a wider distribution, but those away from the terminal are not activated.

This work was supported in part by USPHS grant NS 16295, NRSA Fellowship NS 07172, and the Irma T. Hirsch Foundation.

Received 1 December 1983; accepted 17 February 1984.

1. Katz, B. *The Release of Neurotransmitter Substances* (Thomas, Springfield, Illinois, 1969).
2. Hagiwara, S. & Byerly, L. A. *Rev. Neurosci.* **4**, 69–125 (1981).
3. Llinas, R. & Sugimori, M. *J. Physiol., Lond.* **305**, 197–213 (1980).
4. Baker, P. F. & Gltisch, H. G. *Phil. Trans. R. Soc. B270*, 389–409 (1975).
5. Horn, R. & Miller, J. J. *J. Neurobiol.* **8**, 399–415 (1977).
6. Katz, B. & Miledi, R. *J. Physiol., Lond.* **203**, 459–487 (1969).
7. Ross, W. N. & Stuart, A. E. *J. Physiol., Lond.* **274**, 173–191 (1978).
8. Edgington, D. R. & Stuart, A. E. *J. Physiol., Lond.* **294**, 433–445 (1979).
9. Brown, J. E. *et al. Biophys. J.* **15**, 1155–1160 (1975).
10. Blaustein, M. P. & Hodgkin, A. L. *J. Physiol., Lond.* **200**, 497–527 (1969).
11. Grinvald, A., Cohen, L. B., Leshner, S. & Boyle, M. B. *J. Neurophysiol.* **45**, 829–840 (1981).
12. Ross, W. N. & Krauthamer, V. *J. Neurosci.* (in the press).
13. Miledi, R. & Parker, I. *Proc. R. Soc. Lond. B212*, 197–211 (1981).
14. Hudspeth, A. J. & Stuart, A. E. *J. Physiol., Lond.* **272**, 1–23 (1977).
15. Baylor, S. M., Chandler, W. K. & Marshall, M. W. *J. Physiol., Lond.* **331**, 139–177 (1982).
16. Hagiwara, S. *Adv. Biophys.* **4**, 71–102 (1973).
17. Schnapp, B. J. & Stuart, A. E. *J. Neurosci.* **3**, 1100–1115 (1983).
18. Kuffler, S. W. & Yoshikami, D. *J. Physiol., Lond.* **244**, 703–730 (1975).
19. Edgington, D. R. & Stuart, A. E. *J. gen. Physiol.* **77**, 629–646 (1981).
20. Gorman, A. L. F. & Thomas, M. V. *J. Physiol., Lond.* **308**, 287–313 (1980).
21. Zucker, R. & Stockbridge, N. *J. Neurosci.* **3**, 1263–1269 (1983).
22. Charlton, M. P., Smith, S. J. & Zucker, R. S. *J. Physiol., Lond.* **323**, 173–193 (1982).

Light-dependent calcium release from photoreceptors measured by laser micro-mass analysis

Walter H. Schröder

Institut für Neurobiologie, Kernforschungsanlage Jülich GmbH, D-5170 Jülich 1, FRG

Gordon L. Fain*

Department of Ophthalmology, Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, California 90024, USA

Yoshikami and Hagins¹ first suggested that calcium is sequestered within membranous disks in the outer segments of vertebrate rods and that the bleaching of visual pigment molecules by light causes the release of Ca from the disks. Once released, the Ca was postulated to bind to Na^+ channels or carrier molecules in the plasma membrane to produce the electrical response. This theory, termed the 'calcium hypothesis', is supported by much evidence^{2–5} but remains controversial, largely because of the difficulty in measuring calcium in rods and of demonstrating light-induced release^{6–14}. Here we describe direct measurements of total rod Ca using a new microprobe method, called laser micro-mass analysis, or LAMMA. Using this technique, we show that rods contain large amounts of Ca concentrated in their outer segments. Physiological levels of illumination produce a graded efflux of rod Ca content, amounting to about 10^4 ions per rhodopsin molecule bleached in dim light. As light does not change the rate of Ca influx, the total Ca content of the rod decreases. In bright light, as much as half the total Ca leaves the rod during only 1 min of illumination.

The retinas of toads (*Bufo marinus*) dark-adapted for 3 h or more were removed either in darkness (with the aid of an IR converter) or in dim red light and were placed photoreceptors upwards on a Millipore filter. In the experiments of Figs 1–3, where Ca was measured only for dark-adapted rods, the dissected retina was immediately shock-frozen and processed as described in Fig. 1 legend. In the experiments of Fig. 4, where

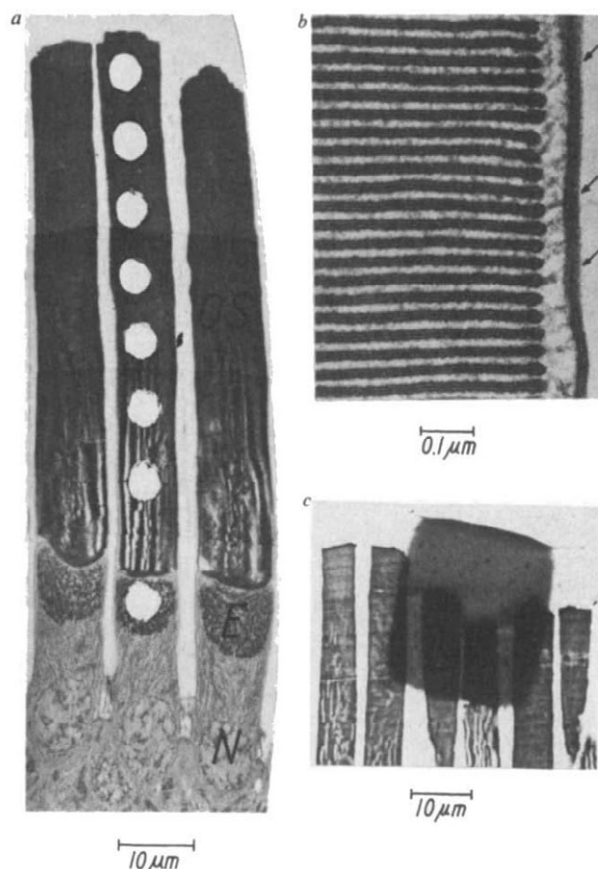


Fig. 1 Morphology and fine structure of rods prepared for LAMMA measurements. *a*, Low-power electronmicrograph of rods showing the holes produced by the high-energy laser of the LAMMA apparatus. OS, outer segment; E, ellipsoid region containing mitochondria; N, nucleus. *b*, High-power electronmicrograph taken from the distal half of the outer segment. Note the fibrillar-like densities between the disks, and the fibrillar connections between the disks and the plasma membrane (arrows; see refs 20, 21). *c*, Calibration of Ca content (see ref. 16). A 600-mesh electron microscope grid was placed directly on top of a section of retina. The section was placed, together with pellets of CaF_2 , in a vacuum beam evaporator at a pressure of $<10^{-6}$ mbar. A film of CaF_2 was then deposited through the holes of the electron microscope grid onto the outer segments to produce a checkerboard-like pattern across the section. The thickness of the film was measured with a quartz crystal monitor (Balzers, QSG, 2010), which in turn was calibrated by weighing a series of standard films on a microbalance.

Methods: The tissue was prepared by rapid immersion in melting Freon at -150°C and then in liquid N_2 . In this figure and in most other experiments the frozen pieces were then transferred to vials containing dry acetone and 0.5% OsO_4 at -194°C and were slowly warmed over the course of several days to permit the acetone gradually to replace the water in the tissue. In a few cases (for example, Fig. 2), the water in the tissue was removed by freeze-drying below -75°C at 10^{-6} – 10^{-7} mbar for 2 days. The tissue was embedded in an Epon-Araldite mixture and sectioned at a thickness of $0.5 \mu\text{m}$ on a Reichert Ultracut ultramicrotome.

the rods were exposed to light, we first measured the electroretinogram from the isolated retina as a control for the light sensitivity of the preparation. We then sliced each retina into eight wedge-shaped pieces. One of these was immediately processed as a control, and the others were exposed to oxygenated Ringer's in darkness and in light for specified periods of time as described in Fig. 4 legend.

Ca content was analysed using a LAMMA-500 (Leybold-Heraeus, Köln, FRG). A complete description of this instrument is given elsewhere¹⁵. Briefly, the LAMMA-500 uses two lasers,

* To whom correspondence should be addressed.

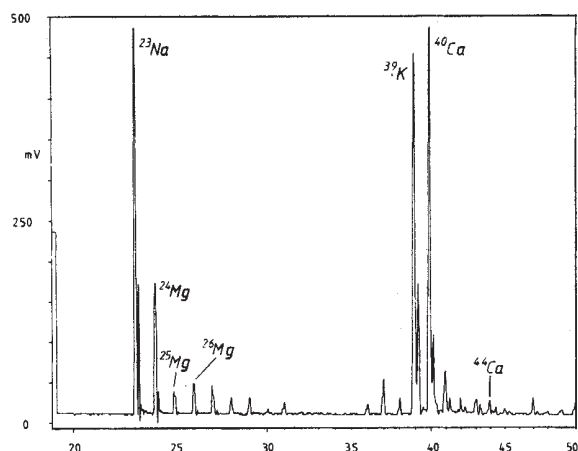


Fig. 2 LAMMA spectrum obtained for a dark-adapted rod outer segment. The spectrum was obtained from a retina shock-frozen immediately after the dissection and freeze-dried (see Fig. 1 legend). Spectra obtained from rods prepared by freeze-substitution were similar.

a low-power He-Ne laser and a high-power Nd:YAG pulse laser, which are colinear. The tissue sections (usually supported on an electron microscope grid) are placed in a vacuum chamber on the stage of a light microscope. The stage is moved to position the low-power laser over a region of interest, and the high-power laser is then activated to vaporize a small area 1–5 μm in diameter. Ions formed during the vaporization are accelerated into the field of an electromagnetic lens and analysed with a time-of-flight mass spectrometer.

Figure 1a illustrates the typical appearance of shock-frozen rods following sectioning and analysis of Ca content. The holes in the rod were produced by the high-energy laser of the LAMMA apparatus. Preservation of the tissue was best in the distal half of the outer segment, as this was directly exposed to the freezing medium (see Fig. 1b). In the proximal outer segment and in the inner segment there was evidence of structural damage, probably due to the formation of ice crystals. However, it was still usually possible to identify the mitochondria forming the ellipsoid region (Fig. 1a, E) and the nucleus (N).

Figure 2 shows the low atomic-weight region of a LAMMA spectrum from a dark-adapted rod outer segment. As the microplasma produced from the tissue by the high-energy laser contains almost exclusively singly-charged particles¹⁵, the m/e ratios in the spectrum give the atomic weights of the analysed mass directly. The peaks for ^{23}Na , ^{39}K and the various isotopes of Mg can be clearly identified, although we cannot, with the procedures presently used, quantify the concentration of these elements in the rods. There is also a prominent peak for ^{40}Ca and a smaller peak for the less common ^{44}Ca (2% natural abundance).

To relate the amplitude of the ^{40}Ca signal to the actual concentration of Ca within the rod, we vacuum-deposited a thin film of CaF_2 directly onto the tissue section¹⁶, as described in Fig. 1 legend. The ratio of the LAMMA signals from rods covered by the thin film and from rods not covered by the film, measured from cells in the same section, was used to determine the total number of Ca atoms within the sampled area. To calculate the calcium concentration in the rod, we also needed to know the thickness of the section. This was determined by vacuum-evaporating platinum on both sides of the section. The section was then re-embedded, resectioned at an oblique angle, and viewed in the electron microscope. The calibration procedure was performed independently on sections of retinæ from two dark-adapted animals, giving $4.4 \pm 1.8 \text{ mmol Ca per l}$ of tissue volume (mean $\pm$ s.d.) in one case and $4.7 \pm 1.8 \text{ mmol l}^{-1}$ in the other. This represents approximately $3\text{--}5 \times 10^9$ Ca atoms per rod outer segment or 1–2 Ca atoms per rhodopsin¹⁷.

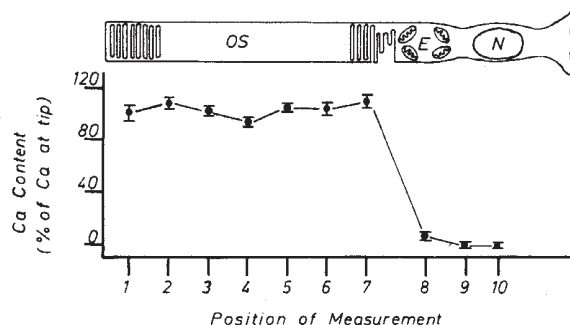


Fig. 3 Distribution of Ca content as a function of distance down the rod. For the outer segment (positions 1–7), seven equally-spaced measurements were made from each of 30 dark-adapted rods. The area within the ^{40}Ca peak at each position was then calculated on a computer after background subtraction. The values for each of the 30 rods were averaged, and the means ($\pm$ s.d.) were expressed as a percentage of the value at position 1. For the inner segment, separate measurements were made in each of seven dark-adapted rods for the ellipsoid (position 8), the cytoplasm between the ellipsoid and the nucleus (position 9) and for the nucleus (position 10). Means ($\pm$ s.d.) of the ^{40}Ca signals are expressed as a percentage of the mean ^{40}Ca signal obtained at position 1 in the outer segment. At positions 9 and 10, the Ca was below the limit of detection of our method ($50 \mu\text{mol l}^{-1}$).

As the LAMMA can produce holes that are only a few μm in diameter (see Fig. 1a), we used this technique to investigate differences in Ca content between different regions of the rod. These measurements (Fig. 3) show that most of the Ca in the rod (>95%) is concentrated within the outer segment. We could detect no significant variation in Ca content along the outer segment, despite large differences in the preservation of the tissue. In the inner segment, Ca could be detected only in the mitochondrial-rich ellipsoid region ($250\text{--}500 \mu\text{mol l}^{-1}$). The decrease in Ca in the inner segment could conceivably have been caused by ice crystal damage in this region. However, we could see no obvious difference in preservation between the innermost outer segment (position 7 in Fig. 3) and the outermost inner segment (position 8), even though the Ca content changed dramatically here in every rod we examined.

Figure 4 shows the effect of light on the Ca content of the rod outer segment. To distinguish efflux from influx in these experiments, we bathed the retinæ in Ringer's in which the normal $^{40}\text{CaCl}_2$ was replaced by $^{44}\text{CaCl}_2$. In darkness, the ^{40}Ca concentration ($\bullet$) of the rod outer segment slowly decreased and the ^{44}Ca content ($\blacklozenge$) slowly increased. These results suggest that, in the dark, ^{44}Ca in the Ringer's slowly exchanges with ^{40}Ca inside the rod. As, however, the decrease in ^{40}Ca appears not to be entirely compensated by the increase in ^{44}Ca , some Ca may also be lost from the rods, perhaps through a gradual deterioration of our isolated retina preparation.

In continuous dim light (68 rhodopsins bleached (Rh^*) per rod per s), the rate of decrease of ^{40}Ca was about three times greater than in the dark. After subtracting the 'dark efflux', the light-induced efflux at this intensity was calculated to be $\sim 8.1 \times 10^5$ Ca atoms per rod per s, or $\sim 10^4$ Ca atoms per Rh^* ; this is in close agreement with the value of the light-induced increase in extracellular Ca measured in toad retina using calcium electrodes¹⁸. As the light intensity was increased, the rate of ^{40}Ca efflux increased. At the brightest intensity we used (10^6 Rh^* per rod per s), the efflux was at least 3×10^7 Ca atoms per rod per s. This is 100 times larger than the efflux in darkness.

We could detect no effect of light on the rate of Ca influx. The rate of ^{44}Ca uptake in steady illumination (Fig. 4, $\diamond$) was indistinguishable from that in darkness within the error of the measurements. The change in total Ca concentration in the light can therefore be obtained directly from the data in Fig. 4 by adding the curve for the ^{44}Ca increase ($\diamond$) to that for the ^{40}Ca decrease at each of the light intensities. These calculations show that light causes a decrease in the total Ca concentration. In

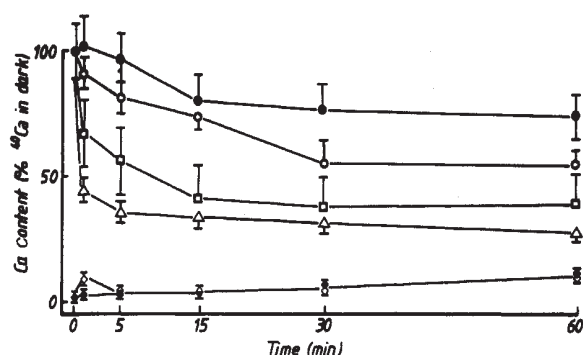


Fig. 4 Effect of light on the Ca content of the rod outer segment. The bars represent 1 s.d. Each symbol represents a different isotope or experimental condition: ●, ^{40}Ca -dark; ◆, ^{44}Ca -dark. The light intensities used for ^{40}Ca were: 68 Rh* per rod per s ○, 8.1×10^3 Rh* per rod per s □ and 10^6 Rh* per rod per s △; and for ^{44}Ca : 10^6 Rh* per rod per s ◇. Illumination was continuous.

Methods: The retina, attached to a Millipore filter, was cut into eight wedge-shaped pieces. One piece was processed immediately as a control. The other pieces were fixed to plates of dental wax with insect pins and placed in 35-mm Petri dishes containing oxygenated Ringer's of the following composition (in mmol l^{-1}): 106 NaCl, 2.5 KCl, 0.13 NaHCO_3 , 1.5 MgCl_2 , 1.8 $^{44}\text{CaCl}_2$, 5.6 glucose, and 3.0 HEPES at pH 7.8. $^{44}\text{CaCl}_2$ was taken from a stock solution prepared by dissolving $^{44}\text{CaCO}_3$ (99% pure; Rohstoffeffuhr, Dusseldorf, FRG) in HCl. The data point at time zero is a control value determined from all retinas used in this figure, expressed as 100%. Other data points give the mean Ca content expressed as a percentage of the control at 1, 5, 15, 30 and 60 min, in each of the experimental conditions. The mean Ca content was calculated from 40 outer segments: 10 measurements were made for each of four pieces of tissue, each from a different retina. The light stimulus was a continuous 503-nm field ~50 nm in diameter. Intensity within the field was uniform within $\pm 10\%$. Absolute values of intensities were measured and numbers of rhodopsins bleached calculated as described in ref. 22.

bright light, one-half to two-thirds of the total Ca in the outer segment disappeared within a few minutes. The remaining one-third to one-half appeared to be unaffected by illumination for as long as our measurements were continued.

The results presented here show that rods in the intact retina contain large amounts of Ca within their outer segments. In the dark, Ca in the rod exchanges slowly with Ca in the external medium. As the permeability of the plasma membrane to Ca seems not to be limiting (W.H.S. and G.L.F., in preparation), the slow rate of exchange may indicate that Ca is sequestered within an intracellular compartment, perhaps the disks. Exposure to light causes as much as half of this Ca to be released, most to be transported out of the rod apparently by Na-Ca exchange^{18,19}. However, as much as 5% of the Ca released from the outer segment accumulates within the mitochondria of the inner segment¹⁹, suggesting that light increases the free Ca concentration within the rod. Our results are therefore consistent with the principal postulates of the Ca hypothesis¹. In addition, our results show that once Ca has left the rod, it is not replaced as long as illumination continues. It will be of interest to examine how the Ca content is restored once the light is extinguished, and to investigate the relationship, if any, between the re-uptake of Ca and the return of photoreceptor sensitivity during dark adaptation.

We thank M. L. Woodruff for his help at the beginning of these experiments; A. Einerhand and J. Lauer for technical assistance; E. Torres and H. Erken for assistance in constructing the photostimulator; D. Schröder and E. Mason for typing the manuscript; H. Kühn and H. Stieve for much useful advice and criticism; H. Stieve, H. Kühn and J. E. Lisman for reading an earlier draft of the manuscript; and H. Stieve and the staff of the Kernforschungsanlage for their hospitality, without which these experiments would not have been possible. This research

was supported in part by a SFB grant 160 to W.H.S. and by NIH grants EY 01844 and EY 00331 to G.L.F. W.H.S. is a Research to Prevent Blindness International Research Scholar.

Received 18 July 1983; accepted 14 March 1984.

- Yoshikami, S. & Hagins, W. A. *Biophys. Soc. Abstr.* **15**, 472 (1971).
- Miller, W. H. *Mechanisms of Photoreceptor Transduction* (Academic, New York, 1981).
- Hubbell, W. L. & Bownds, M. D. *A. Rev. Neurosci.* **2**, 17-34 (1979).
- Fatt, P. *FEBS Lett.* **149**, 159-166 (1982).
- George, J. S. & Hagins, W. A. *Nature* **303**, 344-348 (1983).
- Hagins, W. A. & Yoshikami, S. *Ann. N.Y. Acad. Sci.* **264**, 314-325 (1975).
- Kaufmann, R. *Scanning Electron Microscopy Vol. 2*, 641-644 (AMF O'Hare, Chicago, 1980).
- Schnetkamp, P. P. M., Daemen, F. J. M. & Bonting, S. L. *Biochim. biophys. Acta* **468**, 259-270 (1977).
- Liebmman, P. A. *Invest. Ophthalm. vis. Sci.* **13**, 700-701 (1974).
- Szuts, E. Z. & Cone, R. A. *Biochim. biophys. Acta* **468**, 194-208 (1977).
- Szuts, E. J. *J. gen. Physiol.* **76**, 253-286 (1980).
- Hess, H. H. *Expl Eye Res.* **21**, 471-479 (1975).
- Noell, G., Stieve, H. & Winterhager, J. *Biophys. Struct. Mechanism* **5**, 43-53 (1979).
- Schnetkamp, P. P. M. *Biochim. biophys. Acta* **598**, 66-90 (1980).
- Vogt, H., Heinen, H. J., Meier, S. & Wechsung, R. Z. *anal. Chem.* **308**, 195-200 (1981).
- Schröder, W. H. Z. *anal. Chem.* **308**, 212-217 (1981).
- Fein, A. & Szuts, E. Z. *Photoreceptors: Their Role in Vision* (Cambridge University Press, 1982).
- Gold, G. H. & Korenbrot, J. I. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5557-5561 (1980).
- Schröder, W. H. & Fain, G. L. *Biophys. J.* **45**, 341a (1984).
- Roof, D. & Heuser, J. E. *J. Cell Biol.* **95**, 487-500 (1982).
- Usukura, J. & Yamada, E. *Biomed. Res.* **2**(2), 177-193 (1981).
- Schröder, W. H. Z. *anal. Chem.* **308**, 212-217 (1981).
- Woodruff, M. L., Fain, G. L. & Bastian, B. L. *J. gen. Physiol.* **80**, 517-536 (1982).

ATP-stimulated interaction between epidermal growth factor receptor and supercoiled DNA

B. Mroczkowski, G. Mosig & S. Cohen

Departments of Biochemistry and Molecular Biology, Vanderbilt University, Nashville, Tennessee 37232, USA

The receptor for epidermal growth factor (EGF) has been identified as a transmembrane glycoprotein that has tyrosine-specific kinase activity¹. The kinase activity of the receptor is enhanced in the presence of EGF (or related peptides), and the phosphorylation of a number of substrates, as well as autophosphorylation of the receptor, has been reported¹. Analogous findings have been described for the insulin receptor and the receptor for platelet-derived growth factor (PDGF)². Thus, a number of hormone receptors and several viral transforming proteins³ appear to share the highly unusual property of tyrosine-specific kinase activity. Nevertheless, the specific relationship between tyrosine kinase activity and the control of cell growth and replication is unknown. It is known that after the initial binding of EGF to the plasma membrane, the hormone together with its receptor is rapidly internalized in endocytic vesicles and the hormone is eventually degraded in lysosomes⁴. It is possible that the function of EGF is simply to stimulate internalization of its receptor, and that as a result of its altered location the receptor is able to phosphorylate a cytoplasmic component or even interact directly with a nuclear component. We now report that the purified receptor for EGF is able to interact with and nick supercoiled double-stranded DNA in an ATP-stimulated manner.

The receptor for EGF was isolated from membrane vesicles of A-431 cells⁵. The final purification steps involved EGF-Affi Gel chromatography, wheat germ lectin-Sepharose chromatography and DNA cellulose chromatography. Figure 1c displays a silver stained 10% SDS-polyacrylamide gel of purified 150,000-170,000 molecular weight (MW) (lane 1) and 150,000 MW (lane 2) receptor preparations isolated from A-431 cells. Incubation of the EGF receptor with supercoiled pBR322 plasmid DNA converts form I DNA (supercoiled) to form II DNA. This conversion is ATP-dependent. Assays of equivalent aliquots from each step in this purification are shown in Fig. 1a. Both the kinase activity and the DNA nicking activity co-purify during the EGF-Affi Gel, wheat germ lectin-Sepharose and DNA cellulose purification steps. The purified EGF receptor also reacts with partially relaxed topoisomers of pBR322

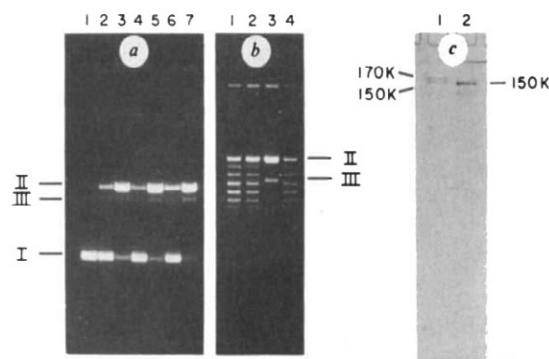


Fig. 1 Agarose gel electrophoresis of pBR322 plasmid DNA after incubation with the EGF receptor in the presence or absence of ATP. **a**, Interaction of supercoiled pBR322 DNA (form I) with various preparations of the EGF receptor. Lane 1, 0.8 μ g of form I DNA; lanes 2 and 3, 0.8 μ g of form I DNA incubated with wheat germ lectin purified EGF receptor in the absence (lane 2) or presence (lane 3) of 5 mM ATP; lanes 4 and 5, 0.8 μ g of form I DNA incubated with wheat germ lectin purified EGF receptor passed through single-stranded DNA agarose in the absence (lane 4) or presence (lane 5) of 5 mM ATP; lanes 6 and 7, 0.8 μ g of form I DNA incubated with wheat germ lectin purified EGF receptor passed through double-stranded DNA cellulose in the absence (lane 6) or presence (lane 7) of 5 mM ATP. **b**, Interaction of partially relaxed preparations of pBR322 (topoisomerase I-treated pBR322 DNA) with the EGF receptor. Lane 1, 0.8 μ g of enzymatically relaxed pBR322 DNA; lanes 2–4, 0.8 μ g of relaxed pBR322 DNA incubated with the EGF receptor in the absence of ATP (lane 2), the presence of 5 mM ATP (lane 3), or the presence of 5 mM AMP (lane 4). **c**, Silver stained 10% SDS-polyacrylamide gel of the 170,000/150,000 MW (lane 1) and 150,000 MW (lane 2) EGF receptor preparations purified by EGF Affi-Gel, wheat germ lectin-Sepharose and DNA-cellulose chromatography.

Methods: Agarose gel electrophoresis was performed in 40 mM Tris-acetate, pH 7.9, 20 mM sodium acetate at room temperature for 12 h at 3 V cm^{-1} . The 0.8% agarose gel (**a**) and 1.0% agarose gel (**b**) were stained with ethidium bromide and photographed with UV illumination. The preparation of A-431 membrane vesicles and subsequent EGF Affi-Gel purification of the EGF receptor has been described elsewhere⁵. The EGF Affi-Gel purified receptor was adsorbed to a wheat germ lectin-Sepharose column and the receptor was eluted with 3 mM *N*, *N'*, *N''*-triethylchitotriose essentially as described for the mouse liver EGF receptor⁶. DNA-cellulose (P-L Biochemicals) chromatography and single-stranded DNA agarose (BRL) chromatography of the wheat germ lectin purified receptor were performed in buffer comprising 0.2% Triton, 10% glycerol, 20 mM HEPES, pH 7.4 and 2 mM MgCl_2 . Approximately one bed volume of wheat germ lectin purified receptor was added to the DNA matrix and adsorption was allowed to take place 30 min on ice with stirring. The receptor preparation did not bind to either matrix. (These DNA columns were used in an attempt to remove any trace nuclease contamination.) The conversion of supercoiled (form I DNA) to nicked DNA (form II DNA) was carried out in 20 mM HEPES, pH 7.4, 5 mM MgCl_2 and 5 mM ATP in a reaction volume of 30 μ l. The reaction was terminated either by the addition of SDS (2%) or by phenol extraction followed by ethanol precipitation. The amount of purified receptor added per reaction mixture varied from 0.2 μ g to 0.8 μ g. The conversion of form I DNA to form II DNA, however, can be detected with as little as 0.02 μ g of purified receptor. In all the experiments described here, incubation was allowed to proceed for 25 min at room temperature and unless specified otherwise the receptor preparation was purified by both wheat germ lectin-Sepharose chromatography and single-stranded DNA agarose chromatography. pBR322 plasmid DNA (derived from *Escherichia coli* strain NH 7007) was purified by Sephacryl S-1000 chromatography followed by caesium chloride buoyant density centrifugation⁸. Wheat germ topoisomerase I (Promega Biotec) was used to partially relax supercoiled pBR322 DNA.

(topoisomerase I-treated preparations of pBR322 DNA) in the presence of ATP to yield form II DNA (Fig. 1b). This conversion does not occur when AMP is substituted for ATP (Fig. 1b, lane 4).

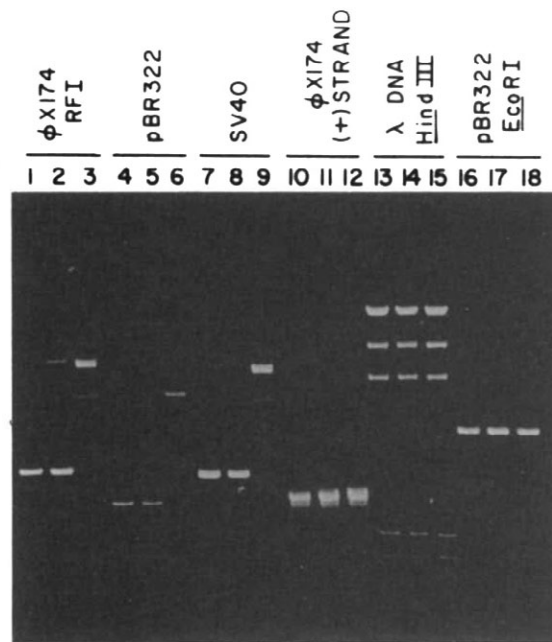


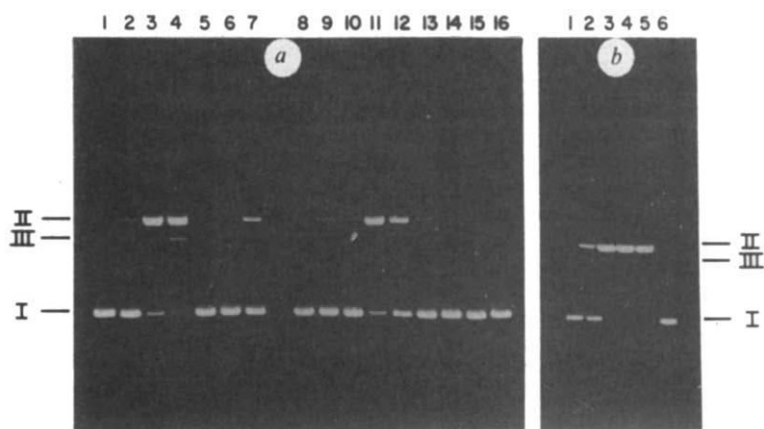
Fig. 2 Specificity of the EGF receptor-DNA interaction. Purified EGF receptor was incubated with different DNA templates in the presence or absence of ATP and the DNA products were analysed by agarose gel electrophoresis. Lanes 1–3, Φ X174 RFI DNA (0.75 μ g) was incubated in the absence of the EGF receptor (lane 1), or in the presence of EGF receptor without (lane 2) or with (lane 3) 5 mM ATP; lanes 4–6, plasmid pBR322 DNA (0.3 μ g) incubated in the absence of the EGF receptor (lane 4), or in the presence of EGF receptor without (lane 5) or with (lane 6) ATP; lanes 7–9, supercoiled SV40 DNA (0.9 μ g) incubated in the absence of receptor (lane 7) or in the presence of receptor without (lane 8) or with (lane 9) ATP; lanes 10–12, Φ X174 single-stranded (+) DNA incubated in the absence of receptor (lane 10), or in the presence of receptor without (lane 11) or with (lane 12) ATP; lanes 13–15, a *Hind*III digest of DNA (1.8 μ g) incubated in the absence of receptor (lane 13) or in the presence of receptor without (lane 14) or with (lane 15) ATP; lanes 16–18, *Eco*RI digest of pBR322 (0.6 μ g) incubated in the absence of receptor (lane 16) or in the presence of receptor without (lane 17) or with (lane 18) ATP. Where indicated, the DNA was digested to completion by the appropriate restriction enzyme (New England Biolabs), phenol extracted and ethanol precipitated prior to addition to the reaction mixture. The DNA products were fractionated on a 1% agarose gel.

The ATP-dependent conversion of supercoiled (form I) pBR322 to form II is observed when either the 170,000/150,000 MW receptor preparations or the 150,000 MW proteolytic cleavage product of the native 170,000 MW A-431 EGF receptor is utilized in the reaction mixture. Both the 170,000 MW and the 150,000 MW forms of the receptor have been shown to possess binding activity for EGF and EGF-stimulated tyrosine-specific kinase activity⁵. The 170,000 MW EGF receptor isolated from normal mouse liver⁶ also possesses the ATP-dependent nicking activity for form I pBR322 DNA (data not shown). It is of interest that in these assay conditions the activity is not significantly altered by the addition of EGF to the reaction mixture; in contrast, EGF enhances the tyrosine kinase activity of the receptor⁵.

The specificity of the interaction of the EGF receptor with DNA was examined by incubating the EGF receptor, in the presence or absence of ATP, with a variety of nucleic acid substrates and examining the reaction products by agarose gel electrophoresis.

As illustrated in Fig. 2, the conversion of form I plasmid DNA to form II DNA is not specific for pBR322; Φ X174 RFI DNA (Fig. 2, lanes 1–3), simian virus 40 (SV40) DNA (Fig. 2, lanes 7–9), and PM2 DNA (data not shown) react in a fashion similar to pBR322 DNA (Fig. 2, lanes 4–6). Regardless of the

Fig. 3 Nucleotide preference and heat stability of the EGF receptor-associated DNA-relaxing activity. **a**, Plasmid pBR322 was incubated with the EGF receptor in standard assay conditions (see Fig. 1) in the presence of various nucleotides. Lanes 1 and 8, pBR322 DNA (0.8 μ g) incubated without EGF receptor; lanes 2–16, pBR322 DNA (0.8 μ g) incubated with the EGF receptor in the absence of nucleotide (lanes 2 and 9) or with 5 mM ATP (lane 3), 5 mM dATP (lane 4), 5 mM 5'-AMP-PNP (lane 5), 5 mM dAMP (lane 6), 5 mM ADP (lane 7), 5 mM TTP (lane 10), 5 mM CTP (lane 11), 5 mM UTP (lane 12), 5 mM GTP (lane 13), 20 mM GTP (lane 14), 5 mM dGTP (lane 15), and 20 mM dGTP (lane 16). **b**, Heat inactivation of the DNA-relaxing activity of the EGF receptor. Receptor preparations were heated in a water bath at the indicated temperature before addition to the reaction mixture. Lane 1, pBR322 DNA (0.8 μ g) incubated in reaction mixture plus ATP but minus the EGF receptor; lane 2, pBR322 DNA incubated with the EGF receptor but minus ATP; lane 3, pBR322 DNA incubated in the presence of both ATP and the EGF receptor; lanes 4–6, pBR322 DNA incubated with ATP and the EGF receptor that had been heated for 15 min at 45 °C (lane 4), 15 min at 65 °C (lane 5), and 5 min at 90 °C (lane 6) before addition to the reaction mixture. Reaction products were fractionated by electrophoresis in 0.8% agarose and stained with ethidium bromide.



source of the closed duplex DNA a small amount of linearized product (form III DNA) is always detected in the presence of ATP and the EGF receptor. In control experiments neither linearized pBR322 (Fig. 2, lanes 16–18) nor a *Hind*III digest of DNA (Fig. 2, lanes 13–15) appeared to be affected by incubation with the EGF receptor in the presence of ATP. Furthermore, using nick-translated ³²P-labelled rabbit pox virus DNA or uniformly ³H-labelled total cellular RNA from vesicular stomatitis virus (VSV) infected cells as substrates we could detect only a trace of nuclease activity (trichloroacetic acid soluble radioactivity) with the former substrate (<0.5% digestion) and none with the ³H-labelled RNA after incubation for 3 h at room temperature in the presence of the EGF receptor and ATP. Thus appreciable enzymatic digestion of these nucleic acids was not detected.

Although the data suggest that the observed activity is intrinsic to or tightly associated with the receptor, we cannot unequivocally rule out the possibility that what we are observing is a low level of ATP-dependent, lectin-adsorbable endonucleolytic contamination which is observed solely in a highly sensitive assay such as the conversion of form I DNA to form II DNA, and which is present in both forms of the EGF receptor prepared from A-431 cells and the receptor prepared from mouse liver. However, no nicking activity was detected when highly purified preparations of tubulin, bovine serum albumin, IgG, EGF or PDGF were assayed.

Some of the biochemical properties associated with the DNA-nicking activity of the EGF receptor are depicted in Fig. 3. Figure 3a demonstrates that both UTP and CTP as well as dATP can substitute for ATP in the reaction mixture. However, the non-hydrolysable analogue of ATP, 5'-AMP-PNP, is not capable of replacing ATP, nor are GTP, dGTP, or TTP effective. The optimum ATP concentration for this reaction is 2–7 mM (data not shown). The activity is extremely heat stable (Fig. 3b), withstanding incubation at a temperature of 65 °C for 15 min and requiring incubation at 90 °C for at least 5 min for complete loss of activity. This thermal inactivation profile is profoundly different from that observed for the tyrosine kinase activity intrinsic to the EGF receptor. Over 80% of the tyrosine kinase activity of the EGF receptor is inactivated by heating for 15 min at 45 °C, while the EGF binding activity of the EGF receptor requires heating for 15 min at 65 °C for inactivation⁷. The observed ATP-dependent DNA reaction appears to have a pH optimum of 6.8–7.6 and is drastically inhibited by relatively low salt concentrations (100 mM KCl or NaCl).

To ascertain whether the reaction products are nicked or whether they represent relaxed, covalently closed circular DNA, agarose gel electrophoresis was performed in the presence or absence of ethidium bromide. In the presence of ethidium bromide the relative migration of a relaxed species of plasmid

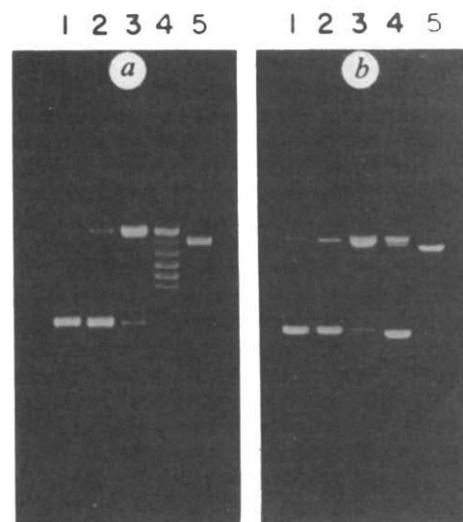


Fig. 4 Comparison of electrophoretic mobilities of pBR322 DNA reaction products in the presence or absence of ethidium bromide. **a**, Agarose gel electrophoresis in the absence of ethidium bromide. Lanes 1–3, pBR322 DNA (0.4 μ g) incubated in the absence of EGF receptor (lane 1), in the presence of EGF receptor without ATP (lane 2), or in the presence of EGF receptor plus ATP (lane 3); lane 4, pBR322 DNA (0.6 μ g) enzymatically relaxed with wheat germ type I topoisomerase; lane 5, pBR322 DNA treated with *Eco*RI. 0.7% agarose gel was electrophoresed in 40 mM Tris-acetate, pH 7.9, 20 mM sodium acetate at room temperature for 12 h at 3 V cm^{-1} . **b**, Agarose gel electrophoresis run exactly as in **a**, except for the inclusion of ethidium bromide (0.5 $\mu\text{g ml}^{-1}$) in both the gel matrix and running buffer. Aliquots of reaction mixtures described in **a** were brought to a final concentration of 0.5 $\mu\text{g ml}^{-1}$ of ethidium bromide before electrophoresis. Lane 1, 0.4 μ g of pBR322; lane 2, 0.4 μ g of pBR322 incubated in the presence of EGF receptor minus ATP; lane 3, 0.4 μ g of pBR322 incubated in the presence of EGF receptor and 5 mM ATP; lane 4, 0.6 μ g pBR322 enzymatically relaxed with wheat germ type I topoisomerase; lane 5, *Eco*RI-treated pBR322. Electrophoresis was performed in the dark to minimize nicking of the DNA.

DNA should change as the molecule progressively binds more ethidium bromide to become increasingly positively supercoiled (compare lanes 4 in Fig. 4a and b) while that of a nicked species should not change in the presence of the intercalating dye. Comparison of lanes 3 in Fig. 4a and b indicates that the reaction product generated in our conditions in the presence of both ATP and the EGF receptor represents a nicked form of plasmid DNA since the relative mobility of form II DNA is not affected by the presence of ethidium bromide.

In view of the biological implications of the observations, and the association of tyrosine kinase activity with both the EGF receptor and pp60^{src}, we have examined three preparations of pp60^{src}. All three preparations generated reaction products apparently identical to those generated by the EGF receptor in the same conditions (data not shown). The DNA-nicking activity of pp60^{src} is also dependent on ATP.

In a number of isolated cases we have observed the generation of a high molecular weight DNA species (possibly representing concatenated DNA) exclusively in the presence of ATP and the EGF receptor. The generation of relaxed topoisomers from covalently closed supercoiled DNA in the presence of pp60^{src} has also occasionally been observed. It is possible that the enzymatic activity reported here is both a nicking and a closing activity reminiscent of other DNA interacting proteins such as DNA topoisomerases. Final proof that this activity is intrinsic to both the EGF receptor kinase and pp60^{src} is not yet available. However the association of DNA-nicking activity with tyrosine kinase preparations from diverse sources is intriguing. A nuclear translocation of these membrane-associated proteins, perhaps via endocytic vesicles and subsequent interaction with DNA could conceivably be responsible for some of the pleiotropic responses accompanying growth stimulation.

We thank Drs R. Erikson and J. Brugge for their gifts of pp60^{src}, Drs S. and R. Moyer for the labelled nucleic acid substrates, and Marty Reich and Katherine Chen for technical assistance. This work was supported by USPHS grants HD-00700 and GM-13221, and American Cancer Society grant BC364. S.C. is an American Cancer Society Research Professor, B.M. is supported by CA-09313.

Received 27 January; accepted 22 March 1984.

1. Cohen, S. in *Biological Response Mediators and Modulators* (ed. August, J. T.) 7-12 (Academic, New York, 1983).
2. Houslay, M. D. & Heyworth, C. M. *Trends biochem. Sci.* **8**, 449-452 (1983).
3. Bishop, J. M. & Varmus, H. in *RNA Tumor Viruses* (eds Weiss, R., Teich, N., Varmus, H. & Coffin, J.) 999-1108 (Cold Spring Harbor Laboratory, New York, 1983).
4. Haigler, H. T. & Cohen, S. *Trends biochem. Sci.* **4**, 132-134 (1979).
5. Cohen, S., Ushiro, H., Stoscheck, C. & Chinkers, M. *J. biol. Chem.* **257**, 1523-1531 (1982).
6. Cohen, S., Fava, R. A. & Sawyer, S. T. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6237-6241 (1982).
7. Carpenter, G., King, L. & Cohen, S. *J. biol. Chem.* **254**, 4884-4891 (1979).
8. Maniatis, T., Fritsch, E. F. & Sambrook, J. in *Molecular Cloning*, 93-94 (Cold Spring Harbor Laboratory, New York, 1982).

Activation of cellular gene by mouse mammary tumour virus may occur early in mammary tumour development

Gordon Peters, Audrey E. Lee & Clive Dickson

Imperial Cancer Research Fund Laboratories, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

There is now good evidence that the induction of mammary carcinomas by mouse mammary tumour virus (MMTV) involves provirus activation of specific cellular genes. Thus, a high percentage of virally induced tumours contain an acquired MMTV provirus in either of two defined integration regions, termed *int-1* and *int-2*, and provirus insertion is accompanied by expression of specific RNA transcripts from these regions¹⁻⁴. We show here that in some recurring, pregnancy-dependent mammary tumours provirus integration within *int-2* has already occurred at the earliest appearance of the tumour and may therefore represent an important step in the development of neoplasia. As judged by the distribution of the acquired MMTV proviruses, the tumours recurring at any one site represent the same clonal population of cells as the original tumour and remain clonal during cycles of proliferation and regression, including the transition to hormone-independent status. These data suggest that either the expression of the *int-2* locus or the function of this putative oncogene must remain responsive to hormones and that some additional event must be responsible for the transition to autonomous growth.

Although MMTV is one of the principal causative agents in the development of mouse mammary tumours, the incidence and progression of the disease is multifactorial, being influenced by such parameters as genetic background, parity and hormonal status of infected animals^{5,6}. In BR6 mice, which suffer a mammary tumour incidence of over 90% as a result of milk-borne MMTV⁷, tumours develop to a detectable size only after an average of four pregnancies and ~70% of them are initially hormone-dependent in that they regress completely or partially between pregnancies^{8,9}. In subsequent pregnancies, the tumour becomes re-established at the same site but eventually progresses to become autonomous and to grow independently of the hormonal status of the animal.

Female BR6 mice were examined at each pregnancy for the presence of detectable tumours and, where possible, surgical biopsies were taken amounting to ~50% of the tumour mass. The aim was to collect repeated biopsies from the same site at each recurrence as the tumour progressed from a hormone-dependent to independent phenotype. As illustrated in Fig. 1, the number of pregnancies required to establish hormone independence was variable, ranging in the examples studied from two at tumour site B to at least six at tumour site A. Figure 1d includes an example of a tumour series from which no biopsies were taken, in order to illustrate the more typical pattern not distorted by the surgical removal of tissue.

DNA of high molecular weight was prepared from each fresh biopsy and analysed by Southern blotting procedures using MMTV-specific probes (see Fig. 2 legend for details). As shown in Fig. 2b, normal tissues in the BR6 mouse (track N) contain three endogenous proviral units of MMTV. Such endogenous sequences are present in all tissues and on both chromosomes, whereas newly acquired MMTV proviruses occur on only one of any pair of chromosomes. Thus, in a truly clonal population of infected cells, the intensity of the hybridization signal from an acquired provirus should be half that of an endogenous unit. In practice, the quantitation can be distorted by the inclusion of any normal tissue in the tumour biopsy, but the data presented here, and in many other studies^{1,2,10-13}, indicate that mammary tumours approach this idealized clonal situation. In all the pregnancy-dependent series examined at least one pair of novel junction fragments was present in DNA from the first biopsy, indicating that acquisition of additional MMTV sequences is probably an early event in tumour induction. Moreover, identically sized, novel fragments were detectable in all subsequent biopsies from the same site, suggesting that recurrence of the tumour at each pregnancy results from expansion of the same, clonal population of tumorigenic cells.

Although the initial pattern of acquired MMTV proviruses remained stable during subsequent cycles of proliferation and regression, DNA from the later biopsies occasionally showed a more complicated pattern (for example tumour sites B and C in Fig. 2). Since the changes were invariably additive, and were not accompanied by loss or rearrangement of established proviruses, they presumably resulted from superinfection of the tumour tissue by MMTV. Judging from the intensities of these additional junction fragments on the autoradiograph, superinfection must have occurred either before or very early in a period of proliferation such that a high percentage of tumour cells contained the same fragments. Since most of the cells in a tumour nodule are presumably lost during regression, each recurrence of the nodule must originate from perhaps a single or, at most, a small number of tumorigenic cells and will therefore only reflect the proviral pattern in this or these cells. It is possible that these represent a stem cell population whose capacity to differentiate in response to the hormonal influences accompanying pregnancy remains unimpaired after infection and possibly 'transformation' by MMTV.

At this stage, we cannot formally exclude the possibility that the acquisition of additional MMTV sequences may have contributed to the transition of tumours to autonomous growth. However, since examples have been found in which hormone independence was apparently achieved without further provirus

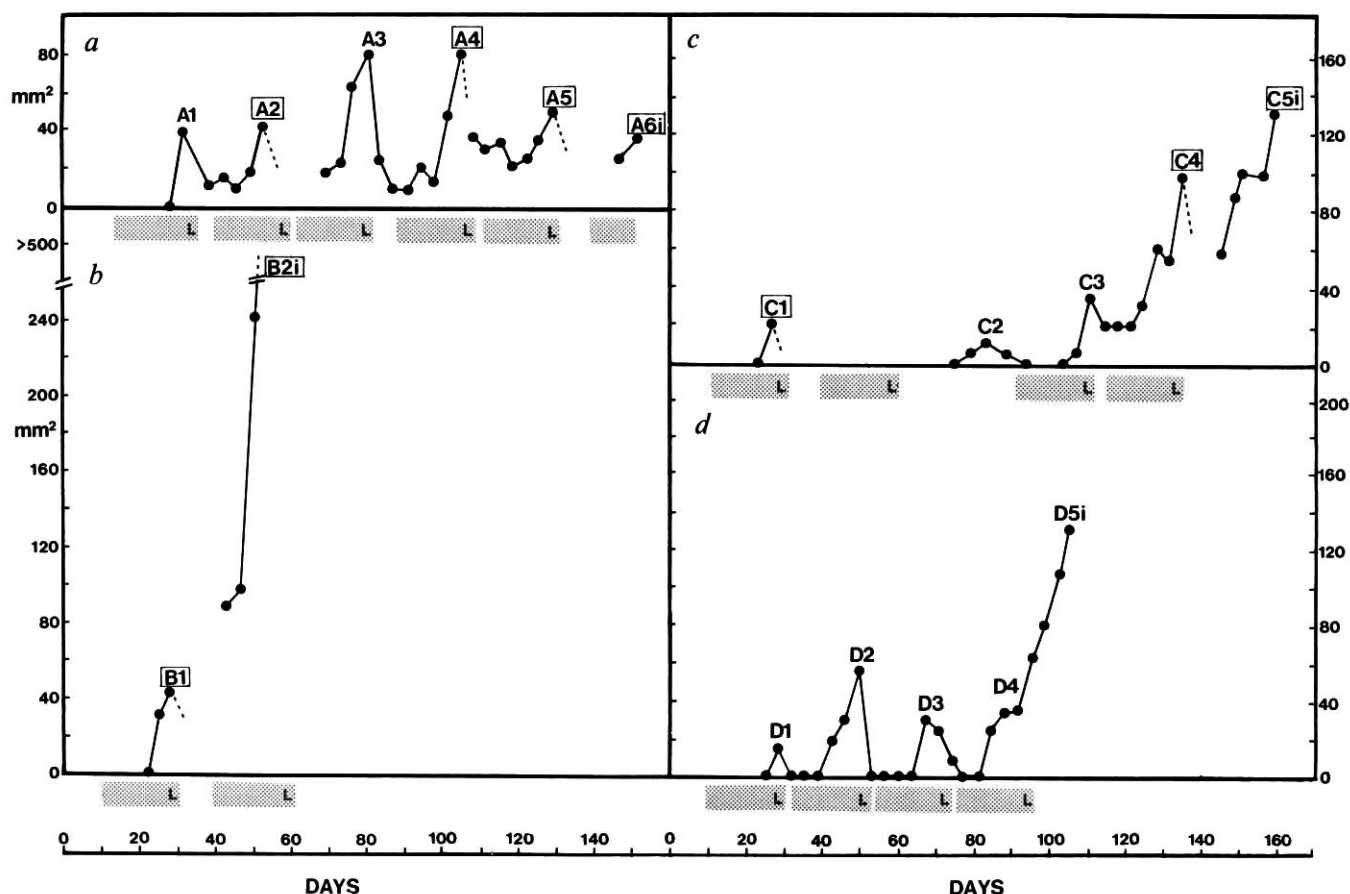
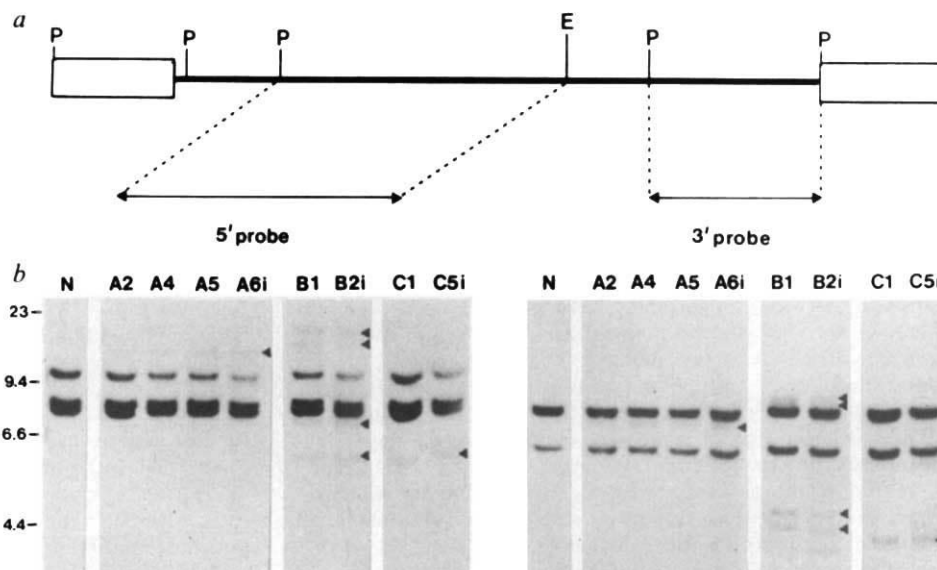


Fig. 1 Cycles of pregnancy-dependent tumour growth and regression. Female BR6 mice were routinely examined for palpable mammary tumours and the progression of each tumour during cycles of pregnancy was monitored. *a-d*, Four independent sites (A-D) at which recurrent tumours arose in four separate mice. The shaded bars below the abscissa of each panel indicate the observed periods of pregnancy, with L signifying delivery of a litter. Size measurements were taken twice weekly, and are expressed on the ordinate as mm², representing the product of two orthogonal diameters⁸. Boxed letters and numbers refer to the site and cycle of growth at which surgical biopsies, amounting to ~50% of the tumour mass, were removed for histological examination⁹ and DNA extraction². No biopsies were taken from site D in order to display a more typical pattern of tumour growth and regression. The symbol i indicates the cycle or biopsy at which the tumour was judged to have become hormone-independent. We are not certain that the tumour designated A6i had fully progressed to hormone independence since the presence of other independent tumours in the same mouse necessitated death at this point.

Fig. 2 Analysis of MMTV proviral units in recurring, pregnancy-dependent mammary tumours. *a*, A restriction map of the GR strain of MMTV provirus is depicted showing the sites of cleavage for the enzymes *Eco*RI (E) and *Pst*I (P)¹⁰. Digestion of cellular DNA with *Eco*RI generates two virus-cell junction fragments distinguishable by hybridization with the cloned 5'-specific and 3'-specific fragments as indicated². *b*, DNA of high molecular weight was prepared² from freshly dissected biopsies of mammary tumours which recurred at three independent sites (A, B and C) in three different animals (see Fig. 1). The numbers refer to cycles of tumour growth, with i signifying the final, hormone-independent tumour. N represents the normal tissue control, in this case the spleen taken from the same animal as the A series of tumours. Samples of DNA (10 µg) were digested with an excess of *Eco*RI, fractionated on 0.8% agarose gels and transferred onto nitrocellulose filters as described previously². The filters were then hybridized for 36-48 h with either the 5' or 3' specific fragment of viral DNA, labelled with ³²P-dCTP by nick-translation. Before autoradiography the filters were washed in 0.1 × SSC/0.1% SDS at 65 °C². The sizes of the various virus-cell junctions detected in this way were estimated relative to fragments of λ DNA generated by digestion with *Hind*III (shown in kilobases on the left of the panel). Normal tissues from BR6 mice contain three endogenous units of MMTV, characterized by 5' junction fragments of 7.7, 7.9 and 9.6 kb, while the 3'-specific junctions appear as a 6.2 kb band and a 7.6 kb doublet. The positions of novel fragments corresponding to acquired MMTV proviruses are indicated (◄).



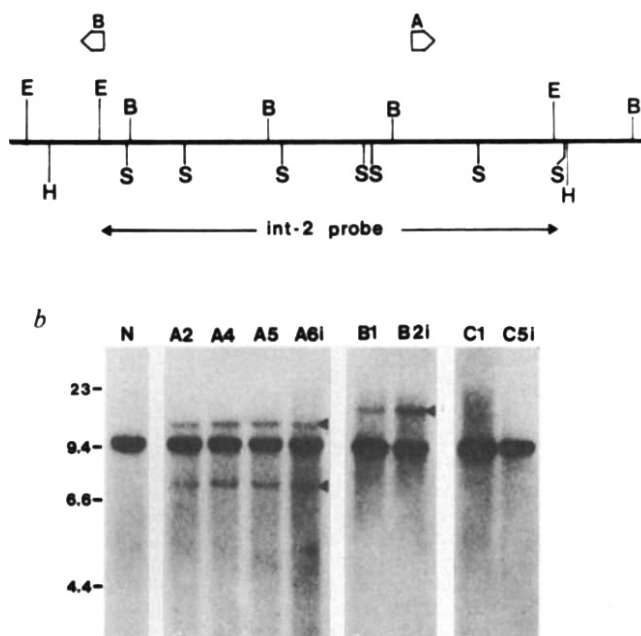


Fig. 3 Provirus insertion in the *int-2* locus in pregnancy-dependent mammary tumours. *a*, The restriction map of the MMTV *int-2* locus in BALB/c DNA indicates the sites of cleavage for the enzymes *EcoRI* (E), *HindIII* (H), *BamHI* (B) and *SacI* (S). The identification and characterization of this locus, and the derivation of the 10.0 kb *EcoRI* fragment used as a probe, have been presented elsewhere^{2,4}. Using these enzymes, no restriction site polymorphisms were detected in the relevant DNAs from BALB/c and BR6 mice. The arrow symbols above the map indicate sites and orientation of integrated proviruses as discussed below. *b*, The nitrocellulose filter carrying the DNA samples analysed with the 3'-specific viral probe in Fig. 2*b* was rinsed in alkali to remove residual probe and then hybridized with the 10.0 kb *int-2* probe indicated. Conditions and symbols are as described in the legend to Fig. 2. N represents a sample of normal tissue DNA, in this case the spleen, showing the homologous uninterrupted 10.0 kb *EcoRI* fragment detected with this probe. The sizes of any novel fragments generated by provirus insertion (identified by the symbol ◀) were estimated relative to *HindIII* digested λ DNA standards as shown. From these data, it was possible to deduce both the site and transcriptional orientation of the provirus integrated within *int-2* in both the A and B tumour series. As indicated in *a*, these two proviruses map about 6-7 kb apart in the cellular DNA and are in opposite transcriptional orientations. In the C series of tumours, no provirus insertions were detected in over 30 kb of DNA centred on the *int-2* locus.

integration (as at tumour site A), we would argue that the acquisition of further proviral elements during progression of the tumour probably reflects fortuitous superinfection and does not contribute to the phenotypic change.

We have previously identified a region of DNA on mouse chromosome 7, *int-2*, in which an MMTV provirus has integrated in ~50% of all BR6 mammary tumours examined whether hormone-dependent or independent^{2,4,14}. As we were interested in determining the status of this locus in the pregnancy-dependent tumour series, the nitrocellulose filters used in preparing Fig. 2 were rehybridized with a probe corresponding to the 10.0 kilobase (kb) *EcoRI* fragment from *int-2* (ref. 2). As indicated in Fig. 3, both the A and B tumour series showed evidence for provirus integration within this fragment. The simplest example is provided by the A series in which two novel *EcoRI* fragments were detected in addition to the normal 10.0 kb fragment present on the uninterrupted chromosome. The larger of these novel fragments was identical in size (~12.5 kb) to the virus-cell junction detected with a 5'-specific viral probe (Fig. 2*b*); while the smaller (~7.4 kb) coincided with the corresponding 3'-specific junction. These data are therefore consistent with integration of a complete MMTV provirus at the position, and in the transcriptional orientation, indicated on the restriction

map in Fig. 3. With the site B tumours only one novel fragment was detected in these analyses, corresponding in size to one of the 5'-specific junctions observed in Fig. 2*b*. The provirus integration site therefore mapped very close to the 5' boundary of the 10.0 kb *EcoRI* fragment, and in the orientation shown (Fig. 3*a*), such that the 3'-specific junction had only a very short region of overlap with the probe. This interpretation has been verified by preparing Southern blots with each of the different restriction endonucleases whose sites are depicted in Fig. 3*a* (data not shown).

Two significant conclusions can be drawn from these observations. First, the A and B tumour series are clonal, in that the same provirus, integrated within *int-2*, remains detectable and unaltered during cycles of pregnancy-dependent growth and regression and in the transition to hormone independence. The second is that since integration within *int-2* apparently occurs by the earliest appearance of the tumour, it may represent an important step in the development of neoplasia. This latter conclusion is supported by data to be presented elsewhere which indicate that polyadenylated RNA transcripts derived from the *int-2* locus are present specifically in tumours which have sustained a provirus integration in *int-2*. Our present interpretation is therefore that MMTV-induced carcinogenesis involves proviral activation of specific cellular genes and that *int-2* represents one such gene.

The observations of provirus insertions in these tumours make significant predictions regarding the involvement of *int-2* in tumorigenesis. If the activation of RNA and protein synthesis from a putative oncogene within *int-2* is the primary event in the onset of neoplasia, then either the expression of this gene product or the target to which its activity is directed, must remain under hormonal control. It is not possible at this stage to determine whether this reflects the direct action of hormones on some functions of the provirus¹⁵⁻¹⁸, or whether the tumour cells are simply continuing to respond normally to the hormonal fluxes accompanying pregnancy^{5,19}. However, the data also imply that activation of *int-2* or other analogous loci are not sufficient for the development of frank malignancy and may therefore represent only part of a multistage process. Some additional stochastic event, such as the activation or mutation of alternative cellular genes, may be required to obviate this hormone dependence and permit the tumour to develop autonomously. Pregnancy-dependent tumours in the BR6 mouse provide a powerful system in which to dissect the roles of these various factors in mammary carcinogenesis.

We thank Ros Smith, Sharon Brookes and Len Rogers for technical assistance; Mike Hayman, Malcolm Parker and John Wyke for helpful discussions and Audrey Gibson for help in preparing the manuscript.

Received 12 December 1983; accepted 5 March 1984.

1. Nusse, R. & Varmus, H. E. *Cell* **31**, 99-109 (1982).
2. Peters, G., Brookes, S., Smith, R. & Dickson, C. *Cell* **33**, 369-377 (1983).
3. Nusse, R., van Ooyen, A., Cox, D., Fung, Y. K. T. & Varmus, H. *Nature* **307**, 131-136 (1984).
4. Dickson, C., Peters, G., Smith, R. & Brookes, S. *Cold Spring Harb. Conf. Cell Proliferation and Cancer* (Cold Spring Harbor Press, New York, in the press).
5. Moore, D. H. et al. *Adv. Cancer Res.* **29**, 347-418 (1979).
6. Cardiff, R. D. & Young, L. J. T. in *Viruses in Naturally Occurring Cancers* (eds Essex, M., Todaro, G. & zur Hausen, H.) 1105-1114 (Cold Spring Harbor, New York, 1980).
7. Foulds, L. *Br. J. Cancer* **3**, 230-239 (1949).
8. Lee, A. E. *Br. J. Cancer* **22**, 77-82 (1968).
9. Lee, A. E., Pang, L. S. C., Rogers, L. A. & Jeffery, R. E. *Clin. exp. Metastasis* **1**, 223-227 (1983).
10. Cohen, J. C., Shank, P. R., Morris, V. L., Cardiff, R. & Varmus, H. E. *Cell* **16**, 333-345 (1979).
11. Cohen, J. C. & Varmus, H. E. *J. Virol.* **35**, 298-305 (1980).
12. Fanning, T. G., Puma, J. P. & Cardiff, R. D. *J. Virol.* **36**, 109-114 (1980).
13. Morris, V. L., Vlasschaert, J. E., Beard, C. L., Milazzo, M. F. & Bradbury, W. C. *Virology* **100**, 101-109 (1980).
14. Peters, G., Kozak, C. & Dickson, C. *Molec. cell. Biol.* **4**, 375-378 (1984).
15. Ringold, G. M. *Biochim. biophys. Acta* **560**, 487-508 (1979).
16. Chandler, V. L., Maler, B. A. & Yamamoto, K. R. *Cell* **33**, 489-499 (1983).
17. Hynes, N., van Ooyen, A. J. J., Kennedy, N., Herrlich, P., Ponta, H. & Groner, B. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3637-3641 (1983).
18. Majors, J. & Varmus, H. E. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5866-5870 (1983).
19. Sluyser, M., Verstraeten, R. A. & Van Nie, R. *Int. J. Cancer* **31**, 217-221 (1983).

Long terminal repeats of human T-cell leukaemia virus II genome determine target cell specificity

Irvin S. Y. Chen, Jami McLaughlin & David W. Golde

Division of Hematology-Oncology, Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024, USA

Human T-cell leukaemias and lymphomas associated with the human T-cell leukaemia viruses (HTLV)¹⁻³ are invariably neoplasms of cells with mature T-lymphocyte phenotype^{4,5}. Epstein-Barr virus-transformed B-lymphocyte lines which are productively infected with HTLV may be isolated from patients with HTLV malignancies^{6,7}, but no non-lymphoid tissues seem to be involved. Here, to investigate the basis for this tissue specificity, we introduced type II HTLV (HTLV-II) into a variety of human cells by infection and also by transfection of recombinant genomes. We found no HTLV-II expression in non-lymphoid tissues although expression and correct initiation of transcription was observed in B and T lymphocytes. Our results using recombinant genomes indicate that the restriction of expression is at least partly due to *cis*-acting functions of the long terminal repeats which lie at each end of the HTLV genome.

Normal human peripheral blood T lymphocytes and Epstein-Barr virus (EBV) transformed B-lymphocyte cell lines were productively infected by HTLV-II by co-cultivation with HTLV-II-infected Mo cells⁷ but attempts to infect cells of non-lymphoid origin were unsuccessful (Table 1). Epithelial/B-lymphocyte hybrid cells (D98/Raji⁸ and D98/HRI⁹) were infected, as shown by the presence of HTLV-II proviral DNA in ~10% of the cells for up to 50 generations, but no viral p19 or p24 antigens were

Table 1 HTLV-II infection by co-cultivation with Mo cells

Cells	HTLV-II genome	Viral antigens p19 and p24
Peripheral blood lymphocytes	+	+
EBV-transformed B cell		
G-B	+	+
WIL-2	+	+
Primary fibroblasts		
R	NT	-
C	NT	-
K	NT	-
Fibroblast cell line (143) ⁸	NT	-
Epithelial/B-lymphocyte hybrid line		
D98/Raji ⁹	+	-
D98/HRI ¹¹	+	-

In every case 5×10^5 cells were co-cultivated with an equal number of lethally irradiated Mo cells⁷. In the case of adherent cells, the Mo cells were washed out after 1 week of co-culture and the cells passed for at least an additional 2 weeks before the cells were fixed for indirect immunofluorescence for viral p19 and p24 antigens and their DNA extracted for hybridization by the method of Southern¹⁸. In the case of nonadherent cells, the cells were co-cultured for 5-6 weeks. Most Mo cells were dead by the second week of co-culture. NT, not tested.

detected by indirect immunofluorescence (Table 1) and co-cultivation of infected hybrid cells with normal human peripheral blood lymphocytes did not result in HTLV-II trans-formation of the normal cells (data not shown).

Since HTLV-II infection by co-cultivation is a relatively inefficient process, probably due to the low titres of infectious virus produced by infected cells, we also used DNA transfection with selectable markers to introduce HTLV-II DNA into cells. We initially tested the ability of an HTLV-II provirus (λ H6,

Fig. 1 HTLV-II RNA sequences in 729 B cells stably transfected with λ H6 DNA. **a**, Serial dilutions of poly(A)-containing RNA prepared from Mo cells; a clone of 729 cells transfected with pH6neo (729(pH6neo)1) and normal 729 cells. The indicated amounts of RNA were spotted onto nitrocellulose filter paper and hybridized²⁴. The location of sequences in the probe is shown in **c** and are derived from cDNA clones pH-1 and pH-13¹³. The probe was purified free of pBR322 sequences so only HTLV-II-specific hybridization would be detected. **b**, S₁ nuclease analysis²⁵ of total RNA from mouse L-cells; HTLV-II infected B cells (J-WIL); 729(pH6neo)1; and Mo cells. The cap site of HTLV-II (nucleotide 314 ± 1) was determined previously²⁰. The position of the probe in the 5' LTR of the λ H6 provirus is indicated below the autoradiogram. The probe is 5' end-labelled at the *Bam*HI site (nucleotide 360²⁰) in the LTR. The expected sizes of the protected fragments are 50, 51 and 52 bases²⁰. The size of *Sau*3A-digested pBR322 fragments are indicated. **c**, The restriction enzyme sites *Hind*III (H), *Bam*HI (B) and *Eco*RI (R) in λ H6 proviral DNA¹³ and flanking human cellular DNA. The orientation of the provirus is indicated by 5' and 3' and the right and left arms of the λ 1059 vector are indicated by λ_r and λ_l respectively. The numbers directly beneath the genome indicate the size in kilobases (kb). The numbers between restriction enzyme sites represent the size of HTLV-II DNA fragments. The location of HTLV-II sequences used in the hybridization probe are shown by bars indicated above the map.

Methods: 10^7 729 cells were transfected by spheroplast fusion²⁶ with a provirus HTLV-II subcloned in the *Eco*RI site of pSV2-neo¹⁰ (pH6neo) and plated at 10^5 viable cells (viability was usually ~50%) per well of a 24-well culture dish. The frequency of G418-resistant cells was about 10^{-5} . Cells resistant to 750 μ g ml⁻¹ G418 were propagated for analysis. Details of the construction of pH6neo are available on request.

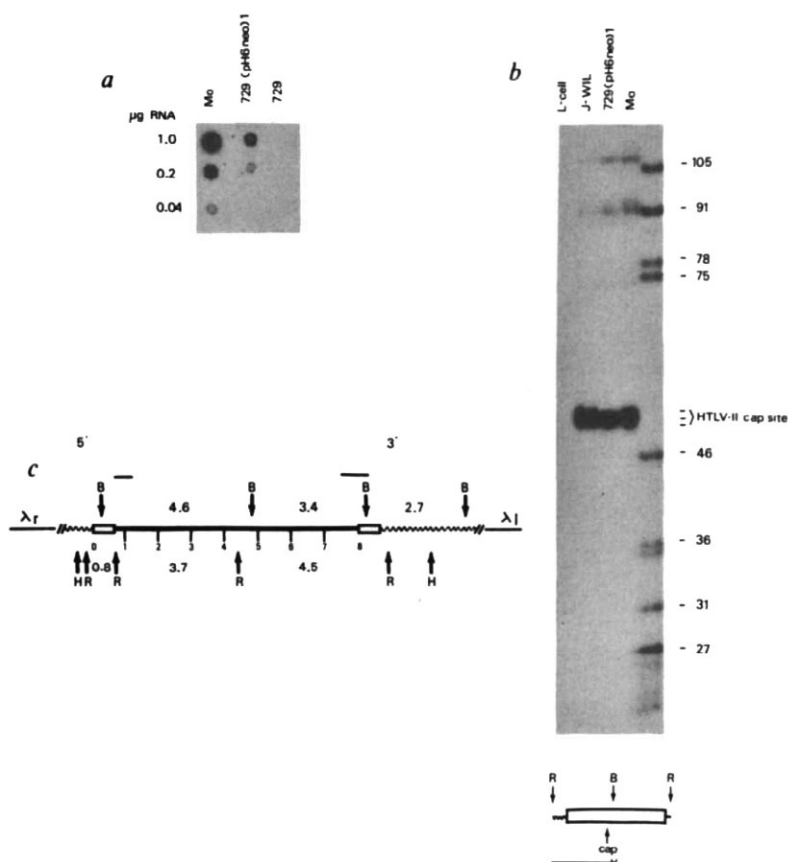


Fig. 1c) to function in an EBV-transformed B-cell line, 729, found to be competent for transfection by protoplast fusion. Cells were transfected with the entire provirus of λ H6 subcloned into pSV2-neo (ref. 10), a recombinant vector selectable in animal cells with the aminoglycoside antibiotic G418.

Approximately 25% of the G418-resistant clones of cells expressed HTLV-II p19 and p24 antigens as assayed by indirect immunofluorescence. Dot blot hybridization of RNA from one of these clones demonstrated expression of HTLV-II RNA (Fig. 1a) and S_1 nuclease analysis showed correct initiation from the cap site of the HTLV-II long terminal repeat (LTR) (Fig. 1b).

To study the restriction of HTLV-II expression in non-lymphoid cells, cells from a fibroblast cell line, 143, derived from a human osteosarcoma and competent for stable transfection¹¹, were transfected with λ H6 DNA. Since 143 cells are deficient in thymidine kinase, we used the co-transfection technique¹² with the herpes simplex virus thymidine kinase gene (pTK). In this way we obtained a stable 143 cell line containing the λ H6 provirus. Restriction enzyme analysis indicates that there are two HTLV-II DNA fragments in the cells, the smaller being the λ H6 provirus with no gross alterations of viral sequences (Fig. 2a). Other clones of 143 cells were obtained by transfection with a molecular clone of an HTLV-II provirus isolated from a transformed cell line, J-LB-II^{7,13}.

The 143 clone containing HTLV-II was negative for viral p19 and p24 antigens as assayed by indirect immunofluorescence. Poly(A)⁺ RNA extracted from the cloned 143 cells did not hybridize to a HTLV-II-specific probe although hybridization could be detected in as little as 1.6 ng of RNA from HTLV-II-transformed Mo cells (Fig. 2b). Therefore, although the complete HTLV-II genome is present in the 143 cells, stable HTLV-II RNA is not detectable.

The lack of HTLV-II expression in several different human epithelial and fibroblast cells containing infected or transfected viral genomes indicates a tissue-specific restriction for HTLV-II transcription. Since the retrovirus LTR contains sequences important for the control of viral transcription and replication^{14,15} we constructed recombinant genomes containing the HTLV-II LTR and the selectable gene *neo*^R from the bacterial transposable element Tn5 used in pSV2-neo¹⁰. The transfection of selectable markers, the expression of which is dependent on *cis*-acting transcription signals, has been used as a simple assay for the efficiency of transcription of various viral and cellular promoters^{16,17}.

Four types of recombinant genomes were constructed to test the transcriptional efficiency of the HTLV-II LTR relative to the SV40 early promoter and SV40 termination sequences (Fig. 3). The first recombinant genome consisted of the *neo*^R gene, transcription of which would be initiated by the 5' LTR, but termination of transcription would occur in SV40 sequences (pJH9LTRneo). The second recombinant represented the reciprocal of the first recombinant, in that the SV40 early promoter would initiate transcription of the *neo*^R gene and termination of transcription would occur in the 3' LTR (pH6SVneoB). The third recombinant would initiate and terminate transcription of *neo*^R in the HTLV-II LTRs (pJH9LTRneoH6LTR). The fourth construct served as a control and consisted of the complete pSV2-neo plasmid inserted into the HTLV-II genome at the same position as for pH6SVneoB (pH6SVneo⁺). Transcription of the *neo*^R gene would be initiated by the SV40 early promoter and terminated by SV40 sequences.

When the recombinant genomes were introduced into the Mo cells by protoplast fusion, G418-resistant cells were obtained at a frequency comparable to that of the SV40 vector, pSV2-neo (Fig. 3). No G418-resistant cells were obtained when Mo cells were fused with pBR322. In pJH9LTRneo and pJH9LTRneoH6LTR, expression of *neo*^R should be dependent upon the initiation of transcription by the HTLV-II LTR. Figure 4 shows an example of one Mo cell clone transfected with pJH9LTRneo. A DNA fragment containing *neo*^R is detected in the Mo cells by Southern hybridization¹⁸. The size of the fragment is consistent with the presence of the entire transcriptional

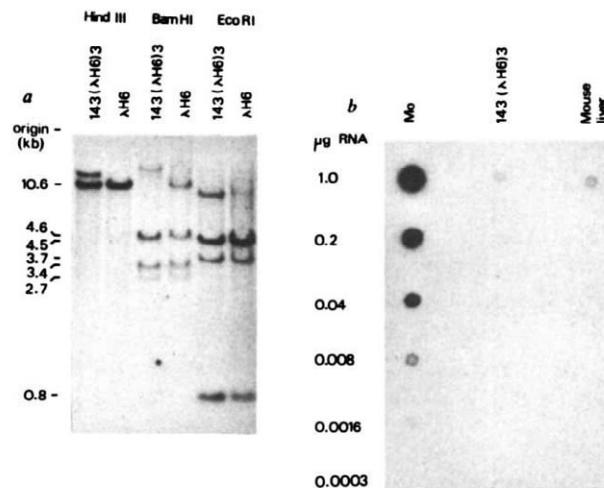


Fig. 2 HTLV-II DNA and RNA sequences in 143 cells stably transfected with λ H6 DNA. **a**, DNA extracted from the tk⁺ transfor- mant 143(λH6)3 digested with *Hind*III, *Bam*HI and *Eco*RI and hybridized by the method of Southern¹⁸ using the probe shown in Fig. 1c¹³. λ H6 DNA is equivalent to 1.0 copy per cell mixed with 15 μ g of normal human DNA and treated in parallel. As *Hind*III does not cleave HTLV-II sequences¹³ the two fragments detected by *Hind*III digestion must represent two HTLV-II DNA fragments present in the cells. The smaller of the two *Hind*III fragments is the same size as the 10.6 kb *Hind*III fragment of λ H6. The larger fragment has lost several of the restriction enzyme sites of λ H6 and contains only a portion of the λ H6 provirus. The HTLV-II DNA fragments indicated in kb are those shown on the map of λ H6 in Fig. 1c. **b**, Poly(A)⁺ RNA prepared from Mo, 143(λH6)3 and mouse liver cells and treated as described in Fig. 1a. Because of the long exposure of the autoradiogram, some nonspecific hybridization to mouse liver RNA is evident.

Methods: 5×10^5 tk⁺ 143 cells¹¹ were co-transfected with a calcium phosphate precipitate of 0.5 μ g pTK, 1 μ g λ H6¹³ and 20 μ g ml⁻¹ calf thymus DNA. Colonies of cells resistant to hypoxanthine-aminopterin-thymidine medium were cloned after 14 days and propagated.

unit of *neo*^R (Fig. 4a). In addition, protection of a 790 nucleotide DNA fragment by S_1 analysis (Fig. 4b) is consistent with initiation of *neo*^R transcription at the cap site of the HTLV-II LTR. Other Mo clones transfected with pJH9LTRneo and pJH9LTRneoH6LTR show the same 5' ends (data not shown). Therefore, transcription of *neo*^R in Mo cells appears to be dependent upon HTLV-II LTR function.

Transfection into 143 fibroblast cells of pSV2-neo, where SV40 sequences are used for initiation and termination of *neo*^R transcription, resulted in approximately 200 colonies of G418-resistant cells per μ g of plasmid (Fig. 3). However, transfection of recombinant genomes where the HTLV-II LTR was necessary for initiation of transcription (pJH9LTRneo, pJH9LTRneoH6LTR) resulted in less than 1/100th the number of colonies. The efficient transfection in 143 cells of pH6SV2neo⁺, where expression of *neo*^R is dependent upon SV40 signals contained between HTLV-II LTR sequences, indicates that viral sequences are not toxic to 143 cells and are not inhibitory to uptake or stable integration of transfected DNA. Therefore, the LTR of HTLV-II appears not to promote the initiation of transcription of the *neo*^R gene in 143 cells effectively.

Note, in addition, that although the SV40 early promoter is functional in 143 cells when transcription is terminated in SV40 sequences (pSV2-neo), the SV40 promoter appears to be incompatible with the 3' HTLV-II LTR when the LTR is substituted for SV40 termination sequences (Fig. 3, pH6SVneoB). In 143 cells this recombinant had a transfection efficiency that was no better than a recombinant with no termination sequences (Fig. 3, pSVneoA-). In Mo cells, pH6SVneoB had a somewhat lower efficiency of transfection than pSV2-neo but higher than

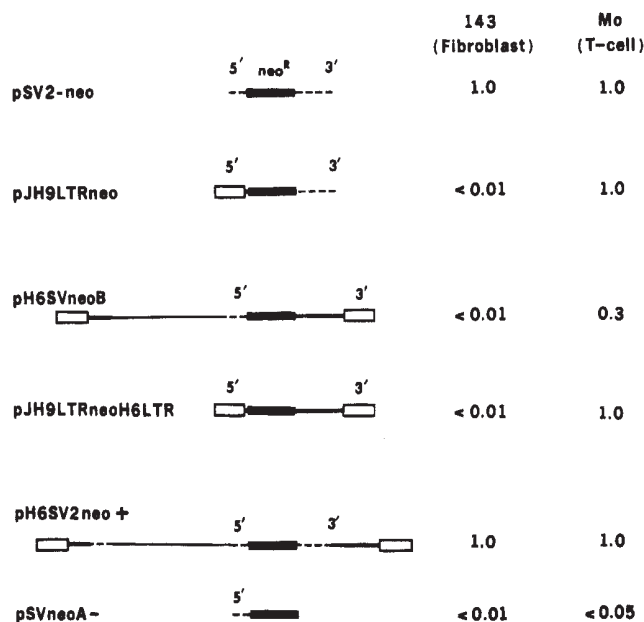


Fig. 3 Relative transfection efficiency of recombinant *neo^R* genes. The different recombinants between SV40, HTLV-II and *neo^R* sequences are shown schematically. HTLV-II sequences are indicated by dark lines; HTLV-II LTR by open boxes; SV40 sequences by dotted lines; *neo^R* sequences by solid bars and pBR322 sequences by thin lines. 5' and 3' indicate the orientation with respect to expected transcription initiation sequences and termination sequences, respectively. The 5' LTR in pJH9LTRneo and pJH9LTRneoH6LTR are joined to the *neo^R* gene by an *EcoRI* site which is located 3 bp from the tRNA^{Pro} primer binding site²⁰. All constructs are propagated as plasmids of pBR322 derivatives. Details of the constructs are available on request.

Methods: 143 cells¹¹ were transfected by the calcium phosphate precipitation method with serial fivefold dilutions of the plasmid DNA. Colonies of cells resistant to 200 $\mu\text{g ml}^{-1}$ G418 were counted after 2 weeks. 10⁷ Mo cells were transfected by the spheroplast fusion method²⁶ and plated at 1.0–3.0 $\times 10^5$ viable cells (viability ~50%) per well of a 24-well culture dish. The number of stably transfected cells resistant to 500 $\mu\text{g ml}^{-1}$ G418 was determined by the proportion of wells in which cells grew after 5–6 weeks of culture. The efficiency of transfection using the SV40 vector pSV2-neo¹⁰, which contains the SV40 early promoter and termination sequences, was about 5×10^{-6} . Because of some variability between transfection experiments performed at different times, the efficiency of transfection was determined relative to pSV2-neo in each experiment.

pSVneoA-. Thus, termination of transcription by the HTLV-II LTR may also be restricted in 143 cells.

Our results demonstrate that in 143 fibroblast cells the HTLV-II LTR functions poorly, if at all, to promote transcription of stable mRNA, in contrast to the Mo cells where the LTR functions efficiently. This pattern parallels that of expression of HTLV-II proviral sequences in 143 and Mo cells (Fig. 1). Note that other retroviruses, Kirsten murine leukaemia-sarcoma virus and simian sarcoma-associated virus, have been shown to replicate in 143 cells¹⁹. Therefore, a *cis*-acting property of the HTLV-II LTR appears to be directly responsible for the lack of HTLV-II expression in the non-lymphoid cells tested.

We recently determined the sequence of the HTLV-II LTR²⁰, and found a sequence of 21 nucleotides, repeated three times in the same orientation, present in U3 upstream from the 'TATAA' box. Although most sequences of the adult T-cell leukaemia virus (HTLV-I) LTR²¹ are distinct from those in HTLV-II, we noted that the 21 nucleotide sequence is highly conserved and repeated in comparable positions of U3. Because this repeated sequence is conserved in regions of the LTR important for transcriptional control, it is possible that this

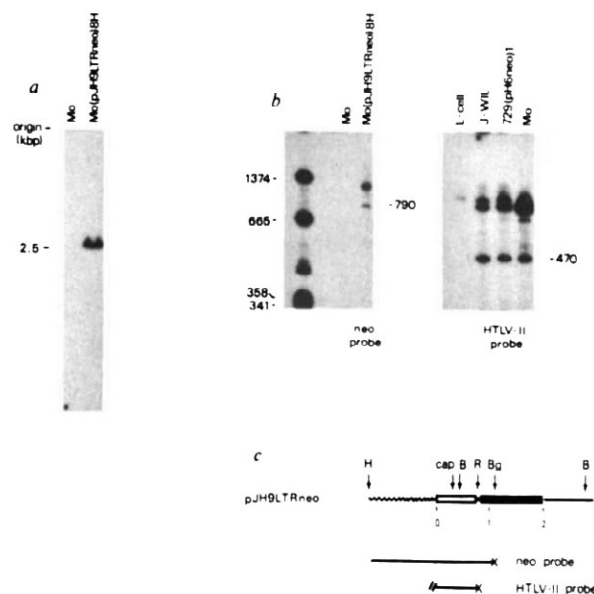


Fig. 4 pJH9LTRneo DNA and RNA sequences in a clone of transfected Mo cells, Mo(pJH9LTRneo)8H. **a**, DNA from Mo(pJH9LTRneo)8H digested with *Bam*HI and hybridized¹⁸ with a ³²P-labelled DNA fragment consisting of only *neo^R* sequences. The DNA fragment of 2.5 kb is the expected size for the *Bam*HI fragment of pJH9LTRneo (see map in **c**). **b**, S₁ nuclease analysis of *neo^R* containing RNA in Mo(pJH9LTRneo)8H (left panel) using a 5' end-labelled *neo^R*-specific probe. S₁ nuclease analysis of HTLV-II RNA (right panel) using an HTLV-II specific probe is shown for comparison. The protected DNA fragments indicated, 790 and 470 nucleotides, are of the size expected for transcription initiation from the cap size of the HTLV-II LTR. The larger protected fragment with RNA from Mo(pJH9LTRneo) results from protection of the entire HTLV-II LTR sequences (beyond the cap site to the 5' end). This protection is a consequence of hybridization of the U3R sequences at the 3' end of HTLV-II RNA to the U3R LTR sequences in the probe thereby further protecting the probe to the 5' end of the LTR. More sensitive mapping with shorter probes showed that the precise 5' end of the LTR is protected (data not shown). Similar larger protected DNA fragments are observed with the HTLV-II specific probe (right panel, middle band). The largest fragment observed with the HTLV-II specific probe is the size of the full length probe, a result of incomplete S₁ digestion and also visible in the L-cell control lane. **c**, Schematic map of pJH9LTRneo. Symbols are as in Fig. 3 except that wavy lines represent human cell DNA sequences. The 5' end-labelled probes for S₁ analysis are shown. The HTLV-II probe is derived from the equivalent region of the 5' LTR of λ H6 (Fig. 1c) up to the 5' *EcoRI* site in human cell DNA. The numbers beneath the map indicate the size in kb. The cap site of HTLV-II is indicated. H, *Hind*III; R, *EcoRI*; Bg, *Bgl*II; B, *Bam*HI.

sequence is required as a *cis*-acting signal for the tissue specificity of LTR function.

The specific transcription of HTLV-II sequences in lymphoid cells resembles the specific transcription of immunoglobulin genes in mouse B lymphocytes^{16,22,23}. Transfected immunoglobulin genes are expressed at low levels in fibroblasts in contrast to their expression in mouse myeloma cells. Sequences related to enhancer sequences of animal viruses have been shown to be responsible for this tissue-specific expression. It is possible that the factors which control the tissue-specific expression of HTLV and immunoglobulin genes represent basic mechanisms for controlling gene expression during T- and B-lymphocyte development respectively. HTLV has apparently evolved in such a manner as to use that mechanism for its pathogenesis in human T lymphocytes.

We thank A. Berk, M. Emerman, J. Gasson, R. Gaynor, K. Shimotohno, W. Wachsman, K. Wilhelmssen and O. Witte for

useful discussions and S. Quan for technical assistance. This work was supported by USPHS grants CA 30388, CA 32737, CA 09297, CA 16042, awarded by the National Cancer Institute, DHHS, and by grant PF-2182 from the American Cancer Society.

Received 17 November 1983; accepted 13 March 1984.

- Poiesz, B. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 7415-7419 (1980).
- Hinuma, Y. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 6476-6480 (1981).
- Kalyanaraman, V. S. *et al. Science* **218**, 571-573 (1982).
- Gallo, R. C. *et al. Cancer Res.* **43**, 3892-3899 (1983).
- Saxon, A., Stevens, R. H. & Golde, D. W. *Ann. intern. Med.* **88**, 323-326 (1978).
- Yamamoto, N., Matsumoto, T., Koyanagi, Y., Tanaka, Y. & Hinuma, Y. *Nature* **299**, 367-369 (1982).
- Chen, I. S. Y., Quan, S. G. & Golde, D. W. *Proc. natn. Acad. Sci. U.S.A.* **80**, 7006-7009 (1983).
- Glaser, R. & Nonoyama, M. *J. Virol.* **14**, 174-176 (1974).
- Glaser, R. & Rapp, F. J. *J. Virol.* **10**, 288-296 (1973).
- Southern, E. M. & Berg, P. *J. molec. appl. Genet.* **1**, 327-341 (1982).
- Bacchetti, S. & Graham, F. L. *Proc. natn. Acad. Sci. U.S.A.* **74**, 590-594 (1977).
- Wigler, M. *et al. Cell* **16**, 777-785 (1979).
- Chen, I. S. Y., McLaughlin, J., Gasson, J. C., Clark, S. C. & Golde, D. W. *Nature* **305**, 502-505 (1983).
- Temin, H. M. *Cell* **27**, 1-3 (1981).
- Temin, H. M. *Cell* **28**, 3-5 (1982).
- Gillies, S. D., Morrison, S. L., Oi, V. T. & Tonegawa, S. *Cell* **33**, 717-728 (1983).
- Chandler, V. L., Maler, B. A. & Yamamoto, K. R. *Cell* **33**, 489-499 (1983).
- Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
- Rhim, J. S., Cho, H. Y. & Huebner, R. J. *Int. J. Cancer* **15**, 23-29 (1975).
- Shimotohno, K., Golde, D. W., Miwa, M., Sugimura, T. & Chen, I. S. Y. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1079-1083 (1984).
- Seiki, M., Hattori, S., Hirayama, Y. & Yoshida, M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3618-3622 (1983).
- Banerji, J., Olson, L. & Schaffner, W. *Cell* **33**, 729-740 (1983).
- Queen, G. & Baltimore, D. *Cell* **33**, 741-748 (1983).
- Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201-5205 (1980).
- Berk, A. J. & Sharp, P. A. *Cell* **12**, 721-732 (1977).
- Schaffner, W. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2163-2167 (1980).

Domain interactions of H-2 class I antigens alter cytotoxic T-cell recognition sites

H. Allen*, D. Wraith†, P. Pala†, B. Askonas† & R. A. Flavell*

* Biogen Research Corp., Cambridge, Massachusetts 02142, USA

† National Institute of Medical Research, Mill Hill, London NW7 1AA, UK

H-2 class I antigens appear to direct the recognition of virus-infected and neoplastic transformed cells by cytotoxic T lymphocytes (CTLs)^{1,2}. Here, to identify the regions of class I antigens involved in CTL recognition, four hybrid class I genes were constructed in which exons were exchanged between the *H-2K^b* and *H-2D^b* genes^{3,4}. These class I genes were expressed in mouse L cells and recognition of the hybrid *K^b/D^b* antigens by CTLs and monoclonal antibodies specific for either *K^b* or *D^b* was investigated⁴. The pattern of CTL and monoclonal antibody recognition obtained indicates three correlations between structure and function of class I antigens. First, most CTL recognition sites and alloantigenic determinants are located on domains 1 and 2 of the antigen molecule. Second, these CTL recognition sites and alloantigenic determinants are not influenced by interaction of domains 1 and 2 with polymorphic regions of domain 3. Third, in contrast, interaction between domains 1 and 2 alters these CTL recognition sites and alloantigenic determinants. The alteration of CTL recognition sites by interaction between domains 1 and 2 suggests that a CTL site may be formed by amino acids from both domains 1 and 2, or that the conformation of amino acids at a CTL site may be altered by interactions between domains 1 and 2. Through these two features, the conformation of CTL recognition sites on H-2 class I antigens may be sensitive to alteration by interaction of either domain 1 or 2 with viral antigens.

The four hybrid class I genes constructed consist of two pairs of reciprocal exon exchanges between the *H-2K^b* and *H-2D^b* genes (Fig. 1a). Thus, exons 2 were exchanged: the resultant constructs of *K^b* exon 2 in the *D^b* gene and *D^b* exon 2 in the

K^b gene encode hybrid *K^b/D^b* antigens consisting respectively of a polypeptide with domain 1 of *K^b* and all other domains from *D^b* (*K^b1/D^b*) and a polypeptide with domain 1 of *D^b* and all other domains from *K^b* (*D^b1/K^b*) (Fig. 1b). Similarly, the promoters and exons 1, 2 and 3 were exchanged: the resultant constructs of the *K^b* promoter and exons 1, 2 and 3 spliced to the remainder of the *D^b* gene and the *D^b* promoter and exons 1, 2 and 3 spliced to the remainder of the *K^b* gene encode hybrid *K^b/D^b* antigens, producing, respectively, *K^b* domains 1 and 2 with all other domains from *D^b* (*K^b1+2/D^b*) and *D^b* domains 1 and 2 with all other domains from *K^b* (*D^b1+2/K^b*) (Fig. 1b). *Ltk⁻* cells were co-transformed with the *K^b*, *D^b* and four *K^b/D^b* hybrid genes and the thymidine kinase (*tk*) gene by CaPO_4 co-precipitation⁵. *Ltk⁺* colonies, selected for by growth in the presence of hypoxanthine-aminopterin-thymidine, were picked and screened by antibody binding for cell-surface expression of *K^b* and/or *D^b* alloantigenic determinants. The cloned cell lines expressing high levels of *K^b*, *D^b* and hybrid *K^b/D^b* antigens were selected for further study.

The presence or absence of *K^b* and *D^b* allodeterminants on the four hybrid *K^b/D^b* antigens was determined by screening these cell lines with a panel of 18 monoclonal antibodies specific for determinants on either *K^b* or *D^b* (Table 1). All these allodeterminants, except one *D^b* allodeterminant, map to domain 1 or 2. The specificities of 12 monoclonal antibodies were mapped to either domain 1 or 2; these locations were basically in agreement with clusters of allodeterminants defined by binding inhibition studies⁶. The *D^b*-specific antibody 28-14-8 mapped to domain 3; the allodeterminant recognized by 28-14-8 was also present on *L^d* and has previously been mapped to domain 3 of this antigen⁷.

The conformation of domains 1 and 2 in the hybrids *K^d1/D^b* and *D^b1/K^b* seems to differ from that in the parental *K^b* and *D^b* antigens. Fifteen out of the 17 parental *K^b* and *D^b* alloantigenic determinants located on domains 1 and 2 are either modified or not present on *K^b1/D^b* and *D^b1/K^b*. Thus, five allodeterminants are absent from either of the two hybrids; these are defined by antibodies K7-38, K9-136 and K10-56.1 specific for *K^b* and H141-29 and K15-25 specific for *D^b*. Ten allodeterminants are present on one or other of these hybrids, but all are modified, as indicated by markedly reduced levels of binding of the respective antibodies to the hybrids compared with *K^b* or *D^b*. The hybrids *K^b1/D^b* and *D^b1/K^b*, however, do retain one and two unaltered allodeterminants respectively, defined by antibody 28-13-3 specific for *K^b* and antibodies 28-14-8 and B22-249.1 specific for *D^b*; this indicates that both antigens are expressed at levels similar to *K^b* and *D^b*.

Recognition of the four *K^b/D^b* hybrid antigens by CTLs was investigated with both *K^b* and *D^b* restricted influenza virus-specific CTLs and *K^b* and *D^b* allospecific CTLs (Table 2, Fig. 2). The pattern of recognition obtained for the two hybrids *K^b1+2/D^b* and *D^b1+2/K^b* indicates first that the majority of both allospecific and virus-specific CTL recognition sites are on domains 1 and 2, and second, that these CTL recognition sites are not influenced by interaction of domains 1 and 2 with polymorphic regions of domain 3; the amino acid sequence of *K^b* differs from *D^b* at 9 residues out of 92 in domain 3 (refs 3, 8). These results for CTL recognition of the hybrids *K^b1+2/D^b* and *D^b1+2/K^b* confirm and extend those obtained for the equivalent *L^d/D^d* hybrids^{9,10}.

In contrast, neither the *K^b1/D^b* nor the *D^b1/K^b* hybrid was recognized by either *K^b* and *D^b* allospecific or *K^b* and *D^b* restricted influenza virus-specific CTLs. This indicates that both allospecific and virus-specific CTL recognition sites, located on domains 1 and 2 of the parental *K^b* and *D^b* antigens, are significantly altered on the *K^b1/D^b* and *D^b1/K^b* hybrid antigens. The cell lines 4-1 (which expresses *K^b1/D^b*) and 3-7 (*D^b1/K^b*) were lysed by an influenza virus-specific and *K^k*-restricted CTL clone (K5)¹¹ when infected with virus (data not shown). The lack of lysis of 4-1 and 3-7 by *K^b* and *D^b* specific CTLs therefore results from failure to recognize the hybrid antigens and not inability of the lines to be lysed.

Table 1 K^b and D^b specific monoclonal antibody binding to hybrid K^b/D^b antigens

Monoclonal antibody	K ^b /D ^b antigen Cell line	Antibody binding (c.p.m. ¹²⁵ I bound)						Location of allodeterminant
		K ^b 1-3	D ^b 2-5	D ^b 1/K ^b 3-7	K ^b 1/D ^b 4-1	D ^b 1+2/K ^b 6-2	K ^b 1+2/D ^b 5-6	
28-13-3		1,036	166	636	68	97	1,541	K ^b 2
K9-178		4,561	136	1,166	115	107	6,279	K ^b 2
K7-309		3,009	97	423	83	61	4,259	K ^b 2
K7-65		2,495	87	326	75	69	3,793	K ^b 2
20-8-4		5,004	96	81	2,308	40	5,829	K ^b 1
B8-3-24		3,880	96	57	1,239	90	5,044	K ^b 1
K7-38		1,203	189	90	217	93	1,931	K ^b 1 and/or 2
K9-136		1,659	69	91	142	64	2,013	K ^b 1 and/or 2
K10-56.1		4,700	89	163	85	55	6,172	K ^b 1 and/or 2
28-14-8		190	4,878	101	3,981	92	6,675	D ^b 3
T17.433		119	5,686	78	2,433	4,757	167	D ^b 2
28-11-5		155	1,859	67	550	1,827	119	D ^b 2
H172-93		114	1,809	81	302	1,459	77	D ^b 2
H166-32		194	2,114	149	325	1,710	148	D ^b 2
H141-31		124	2,073	87	292	1,622	86	D ^b 2
B22-249.1		189	5,745	4,180	86	5,674	55	D ^b 1
H141-29		97	639	72	115	671	63	D ^b 1 and/or 2
K15-25		144	340	124	118	317	109	D ^b 1 and/or 2

The K^b and D^b specific monoclonal antibodies have been characterized previously^{6,13,14}. The antibody binding assay was performed in Falcon flat-bottom 96-well microtitre plates. 10⁵ cells were added to each well and incubated overnight at 37 °C in Dulbecco's modified Eagle's medium supplemented with glutamine, hypoxanthine, aminopterin, thymidine, penicillin, streptomycin and 10% fetal calf serum. The cells were washed with phosphate-buffered saline containing Ca²⁺, Mg²⁺ and 1% bovine serum albumin, and incubated with 50 µl per well of a 5× dilution of hybridoma supernatant for 60 min at 4 °C. After washing, the cells were incubated for 60 min at 4 °C with 50 µl per well of ¹²⁵I-labelled goat anti-mouse IgG containing 100,000 c.p.m. The cells were washed extensively and lysed with 1% Triton X-100; supernatants were collected and radioactivity was determined in a γ counter. Results are expressed as c.p.m. ¹²⁵I bound; each result is the average from triplicate samples and the variation in all cases was not >7.5%. The binding of an irrelevant monoclonal antibody, specific for the haemagglutinin of influenza virus A/PR8, was <150 c.p.m. on all six cell lines. All 18 K^b and D^b specific antibodies gave binding of <150 c.p.m. to a Ltk⁺ cell line 8-1, which was obtained by transfection of Ltk⁺ cells with the *tk* gene alone. Three antibodies that react with determinants on H-2^k class I antigens, H142-23.3, H142-45 and Y-3 (ref. 13), gave binding to the cell line 8-1 of 4,387, 551 and 6,147 c.p.m., respectively. This suggests that the level of expression of K^b, D^b and the four hybrid K^b/D^b antigens is similar to that of endogenous H-2^k class I antigens of the L cells.

Thus, the pattern of CTL and monoclonal antibody recognition obtained for the two hybrid antigens, K^b1/D^b and D^b1/K^b, indicates that interactions between domains 1 and 2 of the hybrids alter parental K^b and D^b CTL recognition sites and alloantigenic determinants located on these domains. Two possible, not mutually exclusive, models would explain the alteration of CTL recognition sites and alloantigenic determinants. First, the sites recognized by CTLs or monoclonal antibodies may be formed by amino acids from both domains 1 and 2. Thus, for example, amino acids from D^b domain 1 that form part of a CTL/antibody site are replaced by different residues from K^b domain 1 in the hybrid K^b1/D^b. Second, the CTL/antibody sites

may be formed by amino acids entirely from one domain. In this case, the conformation of a CTL/antibody site on D^b domain 2 in the hybrid K^b1/D^b, for example, would be altered by interaction of this domain with K^b domain 1. Thus, K^b differs from D^b at 13 amino acid residues out of 90 in domain 1 and at 20 residues out of 92 in domain 2 (refs 3, 8); these differences between K^b and D^b in domains 1 and 2 result in alteration of CTL/antibody sites on the hybrid antigens K^b1/D^b and D^b1/K^b.

Sherman¹² has proposed that H-2 restriction of CTLs may represent the recognition of conformational alterations of class I antigens resulting from interaction with viral antigens. Our report suggests that the conformation of a CTL recognition site

Table 2 Polyclonal allospecific and influenza virus-specific CTL recognition of hybrid K^b/D^b antigens

K ^b /D ^b antigen Cell line	% Specific ⁵¹ Cr release (K:T=20:1)					
	K ^b 1-3	D ^b 2-5	D ^b 1/K ^b 3-7	K ^b 1/D ^b 4-1	D ^b 1+2/K ^b 6-2	K ^b 1+2/D ^b 5-6
CTLs						
Anti-A/X31 + D ^b	-5	33	-4	7	22	2
Anti-A/X31 + K ^b	18	1	-4	2	-1	13
Anti-allo D ^b	-3	27	0	-3	24	2
Anti-allo K ^b	34	-2	5	-2	-5	19

D^b and K^b restricted CTLs specific for influenza virus A/X31 were obtained from spleens of B10.HTG (K^dD^b) and B10.A(5R) (K^bD^d) mice respectively primed by intravenous injection with 10⁷ A/X31 infected syngenic spleen cells. Allospecific CTLs were generated by *in vitro* stimulation of BALB/c (K^dD^d) spleen cells twice with irradiated (3,000 rad) B10.A(5R) (K^bD^d) for anti-K^b or twice with irradiated B10.HTG (K^dD^b) and once with irradiated B10.BYR (K^dD^b) spleen cells for anti-D^b. Ltk⁺ target cells infected with influenza virus were prepared as follows: the cells were trypsinized and plated overnight on bacteriological Petri dishes, for easy resuspension, before infection with A/X31 and ⁵¹Cr-labelling for 1 h at 37 °C. The conditions for infection of the targets and the protocol for the 6-h cytotoxicity assay were as described previously¹⁵. The % virus-specific ⁵¹Cr release was calculated by subtraction of ⁵¹Cr release from uninfected targets. The % allospecific ⁵¹Cr release was calculated by subtraction of ⁵¹Cr release from a Ltk⁺ cell line 8-1, which was obtained by transfection of Ltk⁺ cells with the *tk* gene alone. A CTL to target ratio (K:T) of 20:1 was used for all four polyclonal populations.

Fig. 1 a, Restriction maps of $H-2K^b$ and $H-2D^b$ genes. **b**, Domain structure of K^b/D^b and four K^b/D^b hybrid antigens. In **a**, E = *EcoRI*; B = *BamHI*; Bs = *BssHII*; H = *HindIII*; X = *XbaI*; Xm = *XmaI*. The restriction sites shown are those used to construct and confirm orientation of exon exchanges. The location of the promoter and exons of the K^b gene are shown². Exons 1, 2 and 3 of the D^b gene are located in the 2,200-base pair (bp) *BamHI* fragment that contains the *BssHII* site. Exons 4, 5, 6, 7 and 8 are located in the adjacent 2,400-bp *BamHI* fragment (unpublished data). The restriction sites used to isolate D^b exon 2 and exons 2 and 3 are not present in the nucleotide sequence of a D^b cDNA clone³, which indicates that these sites are located in introns of the D^b gene. In **b**, 1, 2 and 3 are external domains 1, 2 and 3 (alternatively designated N, C1 and C2, respectively¹⁶); TM, transmembrane segment; C, cytoplasmic domain; β_2M , β_2 -microglobulin. H-2 class I antigens are cell-surface glycoproteins of molecular weight (MW) 45,000 associated noncovalently with β_2 -microglobulin, MW 12,000. The class I polypeptide can be divided on the basis of its primary structure into three external globular domains, each of about 90 amino acids, a transmembrane segment of ~40 residues, and one cytoplasmic domain of 30 residues¹⁶. The diagrammatic representation of the H-2 class I antigen is supported by the observation that domains 1 and 2 are stable in isolation and that domain 3 is associated with β_2 -microglobulin¹⁷.

Methods: The four hybrid K^b/D^b genes shown in **a** were constructed as follows: (1) Exchange of exon 2 between D^b and K^b . The K^b and D^b genes were subcloned in the *EcoRI* and *HindIII* sites of pBR327, respectively. The K^b and D^b exons 2 were isolated as *XmaI* 330- and 390-bp fragments, respectively; these fragments contain the *BssHII* site. The D^b *XmaI* 390-bp fragment was inserted directly into a K^b gene subclone, linearized with *XmaI*, from which both *XmaI* fragments of 330 and 50 bp had been deleted. This deletion was obtained by complete digestion of the K^b subclone with *XmaI* and ligation at dilute concentration of DNA to favour recirculation of the vector minus the two fragments. The K^b *XmaI* 330-bp fragment was inserted first into a D^b 5' gene subclone of the 2,200-bp *BamHI* fragment, linearized with *XmaI*, from which the *XmaI* fragment of 390 bp had been deleted. This deletion was obtained by complete digestion of the D^b 5' gene subclone with *XmaI* and ligation at dilute concentration of DNA. Second, a 1,200-bp *XbaI*-*BssHII* fragment was isolated from the D^b 5' gene subclone and inserted in the complete D^b gene subclone, linearized by partial digestion with *XbaI* and complete digestion with *BssHII*. (2) Exchange of promoter and exons 1, 2 and 3 between K^b and D^b . The K^b and D^b promoters and exons 1, 2 and 3 were isolated from the subclones of the two genes as *XbaI* fragments of 1,800 and 1,850 bp, respectively. The corresponding K^b and D^b 3' gene *XbaI* fragments of 8,000 and 11,500 bp, respectively, were isolated from the same digests; these fragments also contain the pBR327 vector sequence. The K^b *XbaI* 1,800-bp fragment was ligated to the D^b *XbaI* 11,500-bp fragment; similarly, the D^b *XbaI* 1,850-bp fragment was ligated to the K^b *XbaI* 8,000-bp fragment. Restriction enzyme digest conditions were as recommended by New England Biolabs. Restriction fragments were separated by electrophoresis in low melting temperature agarose gels; fragments were isolated by melting gel slices at 65 °C and phenol extraction. Purified fragments were ligated with T4 DNA ligase in 50 mM Tris pH 8.0, 7.5 mM MgCl₂, 1 mM ATP and 10 mM dithiothreitol for 3 h at 15 °C. Vector fragments were first treated with calf intestinal phosphatase in 50 mM Tris pH 9.0, 1 mM MgCl₂, 0.1 mM ZnCl₂ and 1 mM spermidine for 1 h at 37 °C. Ligated DNAs were introduced into CaCl₂-treated *Escherichia coli* DH1 or HB101 cells¹⁸. Colonies were picked and constructs were identified by restriction enzyme digests on plasmid mini-preps¹⁸. DNA for transfection of Ltk⁻ cells was isolated from plasmid maxi-preps¹⁸; exon exchange and correct orientation of insertion were confirmed by restriction mapping.

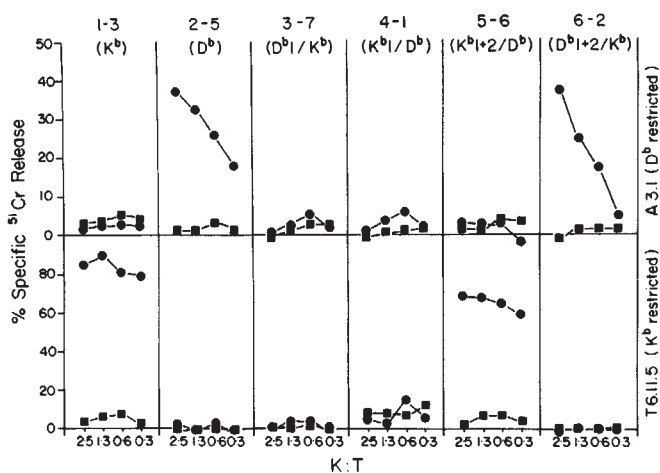
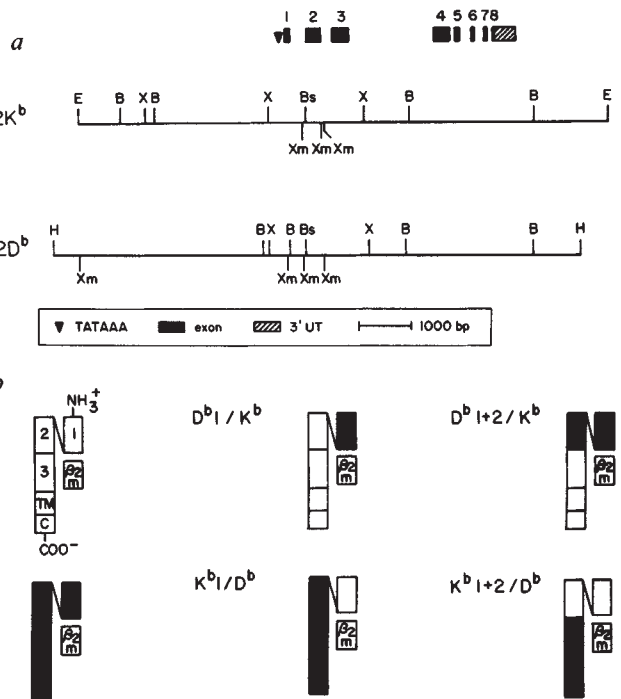


Fig. 2 Cloned influenza virus-specific CTL recognition of hybrid K^b/D^b antigens. The A/X31 influenza virus-specific CTL clones A3.1, D^b-restricted, and T6.11.5, K^b-restricted, were selected and maintained with antigen and T-cell growth factor as described previously¹⁹. Target cells were prepared and the 6-h cytotoxicity assay was performed as described in Table 2 legend. ●, A/X31-infected target cells; ■, uninfected target cells; K:T, CTL to target ratio. The upper graphs show lysis by D^b-restricted clone A3.1; the lower graphs show lysis by K^b-restricted clone T6.11.5.

may be sensitive to alteration by interaction of either domain 1 or 2 with a viral antigen.

We thank Gunter Hammerling, Keiko Ozato and David Sachs for supplying monoclonal antibodies; Susan Calvin and Pat Taylor for assistance with cell culture; and Madlyn Nathanson for assistance in preparing the manuscript. This work was supported by Biogen N.V.

Received 23 January; accepted 19 March 1984.

1. Zinkernagel, R. M. & Doherty, P. C. *Adv. Immun.* **27**, 51-177 (1979).
2. Shearer, G. M. & Schmitt-Verhulst, A. M. *Adv. Immun.* **25**, 55-91 (1977).
3. Weiss, E. *et al. EMBO J.* **2**, 453-462 (1983).
4. Mellor, A. *et al. Nature* **298**, 529-534 (1982).
5. Wigler, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 1373-1376 (1979).
6. Hammerling, G. J., Rusch, E., Tada, N., Kimura, S. & Hammerling, U. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4737-4741 (1982).
7. Evans, G. A., Margulies, D. H., Shykind, B., Seidman, J. G. & Ozato, K. *Nature* **300**, 755-757 (1982).
8. Reyes, A., Schold, M. & Wallace, R. *Immunogenetics* **16**, 1-9 (1982).
9. Ozato, K., Evans, G. A., Shykind, B., Margulies, D. H. & Seidman, J. G. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2040-2043 (1983).
10. Reiss, C. S., Evans, G. A., Margulies, D. H., Seidman, J. G. & Burakoff, S. J. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2709-2712 (1983).
11. Wraith, D. C., Holtkamp, B. & Askonas, B. A. *Eur. J. Immun.* **13**, 762-766 (1983).
12. Sherman, L. A. *Nature* **297**, 511-513 (1982).
13. Ozato, K. & Sachs, D. H. *J. Immun.* **126**, 317-321 (1981).
14. Lemke, H., Hammerling, G. J. & Hammerling, U. *Immun. Rev.* **47**, 175-206 (1979).
15. Zweierink, H. J., Courtneidge, S. A., Skehel, J. J., Crumpton, M. J. & Askonas, B. A. *Nature* **267**, 354-356 (1977).
16. Coligan, J. E., Kindt, T. J., Uehara, H., Martinko, J. & Nathanson, S. G. *Nature* **291**, 35-39 (1981).
17. Yokoyama, K. & Nathanson, S. G. *J. Immun.* **130**, 1419-1425 (1983).
18. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning (A Laboratory Manual)* (Cold Spring Harbor Laboratory, New York, 1982).
19. Townsend, A. R. M., Taylor, P. M., Mellor, A. L. & Askonas, B. A. *Immunogenetics* **17**, 283-294 (1983).

Two mechanisms of expression of a predominant variant antigen gene of *Trypanosoma brucei*

Peter Myler*, Richard G. Nelson†, Nina Agabian† & Kenneth Stuart*

* Issaquah Health Research Institute, 1595 NW Gilman Blvd, Issaquah, Washington 98027, USA

† Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA

African trypanosomes evade the host immune response by periodically switching their variant surface glycoprotein (VSG) coat¹⁻³. The resulting, serologically distinct variant antigenic types (VATs) appear in a loosely ordered sequence and those arising early in infections are termed predominant VATs⁴⁻⁸. VAT switching reflects the successive transcriptional activation of single VSG genes from a large repertoire. Activation of some VSG genes is accomplished by duplication of a previously silent basic copy (BC) gene and insertion of this expression linked copy (ELC) near a chromosomal telomere where it is expressed⁹⁻¹⁶. However, other VSG genes, always located near a telomere, use a non-duplication activation (NDA) mechanism¹⁶⁻¹⁹. We report here that the gene encoding the predominant IsTat 1.A VSG can be activated with or without duplication. Four of six independently derived clones activated the 1.A gene without gene duplication or detectable rearrangement. The other two contained an active 1.A ELC, possibly generated by a gene conversion extending to the end of the telomere. The ability to utilize both NDA and ELC mechanisms of gene activation and perhaps an alternative mechanism for gene duplication may account for the fact that VAT 1.A is the most predominant VAT of the IsTat 1 serodeme.

Derivation of the IsTat 1 serodeme has been described previously²⁰. Many immune competent rats, mice or *Peromyscus* singly infected with different IsTat 1 VATs produced first relapse populations that were invariably 100% VAT 1.A. Thus, 1.A is the most predominant VAT of the IsTat 1 serodeme. Each of six independently derived 1.A clones (Fig. 1) reacted with four monoclonal antibodies which recognize different epitopes of the 1.A VSG molecule (P.M., unpublished results), indicating that they all expressed the same VSG. The mechanism of 1.A VSG gene activation in these clones was examined by genomic Southern analyses. Figure 2a shows an autoradiogram of *SalI*-digested DNA from five clones which do not express 1.A VSG (1.D, 1.3, 1.5, 1.7, 1.11), five 1.A clones from the first relapses of each of these (1.A¹, 1.A³, 1.A⁵, 1.A⁷, and 1.A¹¹), a subclone of the original IsTat 1.A (1.Ae), and two clones derived from a first relapse population of IsTat 1.A (1.A¹ and 1.X^A). The VAT is indicated after the point (for example, 1.Ae), the parent VAT is indicated by a superscript, and a lower case letter is used to designate clones when needed. The 1.A VSG cDNA probe, which contains one *SalI* site (Fig. 2d) also present in each genomic copy of the gene, hybridizes to four *SalI* fragments in 1.Ae and 1.A⁷ and to three fragments in each of the other clones (Fig. 2a). The band at 1.1 kilobases (kb) is composed of co-migrating fragments extending from the internal *SalI* site to another *SalI* site present at the same location 5' to each 1.A gene. The remaining *SalI* fragments contain the 3' ends of these genes and allow determination of the number of 1.A genes present in each clone. Like the original VAT 1.A (1.Ae, 15 and 16), the relapse clone 1.A⁷ contains a 1.A ELC. However, no 1.A ELC is present in four other relapse-derived 1.A clones (1.A¹, 1.A³, 1.A⁵ and 1.A¹¹). The latter clones, their parent VATs (1.1, 1.3, 1.5 and 1.11) and all other heterologous VATs we have examined (for example, 1.7, 1.X^A and 1.1^A) contain only two copies of the 1.A gene. One copy, present on a 13.5-kb *SalI* fragment, is located in a non-telomeric region and has been cloned in λ_{1059} and extensively mapped (Fig. 4). The other copy is located near a telomere, as evidenced by its size variation among VATs (5-8 kb, Fig. 2a) and the presence of a 'universal

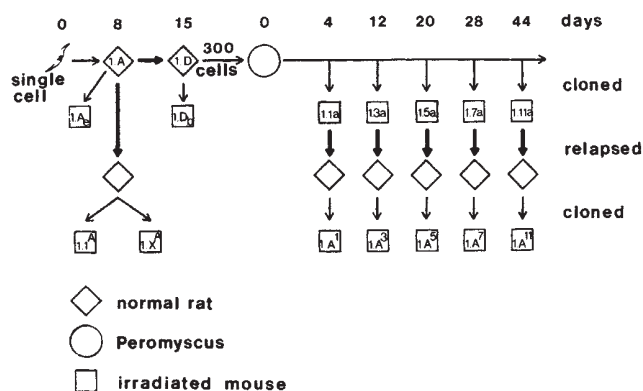


Fig. 1 The IsTat 1 serodeme²⁰ was initiated with a single trypanosome cloned from a population of which ~70% of the cells expressed 1.A. The first parasitaemia (day 8) population was designated IsTat 1.A. Three hundred cells of the first relapse population (1.D, day 15) from the same immune competent rat, were injected into a deer mouse, *Peromyscus leucopus*. Samples were taken at 4-day intervals, expanded in lethally irradiated mice and then cloned to obtain the VATs shown. Clone 1.Db expresses the same VSG as 1.1a (ref. 15) and thus VAT 1.1 cells, were derived from the growth of VAT 1.D cells without an intervening antigenic switch. Approximately 5-500 trypanosomes from each of the six VATs were injected into immune competent rats and the resulting first relapse populations subsequently cloned. The relapse populations from VATs 1.1, 1.3, 1.5, 1.7 and 1.11 were 100% VAT 1.A re-expressors as determined by their reaction with anti-1.A VSG monoclonal antibodies. The relapse population from 1.A yielded two clones expressing 1.1 VSG (from their reaction with anti-1.1 VSG monoclonal antibodies) and one clone expressing a VAT (1.X^A) not recognized by any of our VAT specific antisera.

restriction site' 3' to the gene (Fig. 4). These data suggest that an ELC is present in some 1.A expressors but absent in others.

An alternative interpretation of the above data is that the extra bands in 1.Ae and 1.A⁷ are not ELCs but are due to discrete heterogeneity in the length of telomeric sequences flanking a single 1.A gene. Similarly, the presence of ELCs in 1.A¹, 1.A³, 1.A⁵ and 1.A¹¹ might be concealed by extensive telomeric size heterogeneity, that is, 'ghost ELCs'²¹. To resolve these issues we digested DNA from each clone with *HincII*, which cleaves within the VSG 1.A coding region (Fig. 2d) and 3' to each of the 1.A genes (Fig. 4). This allows analysis of the number of 1.A genes independently of telomeric length variation. The 2.0- and 1.3-kb *HincII* fragments present in every DNA shown in Fig. 2b contain the 3' ends and the immediate 3'-flanking sequences of the non-telomeric and telomeric 1.A genes, respectively. Densitometric scans (Fig. 2c) show a doubling of intensity of the 1.3-kb band compared with the 2.0-kb band, in 1.Ae and 1.A⁷ but not in the other clones. This confirms the presence of a 1.A ELC in these two clones and its absence in the others, and shows that the other telomeric 1.A gene acted as the BC.

The single telomeric 1.A gene in 1.A¹ is preferentially sensitive to DNase I (Fig. 3), illustrating that the telomeric BC is transcribed in those clones using the NDA mechanism. It is not preferentially DNase I sensitive in non-1.A expressors (P.M., data not shown). In 1.A⁷ (and 1.Ae, not shown), only the ELC is DNase sensitive (Fig. 3b), indicating that the duplicated copy and not the telomeric BC is transcribed. Our results therefore show that IsTat 1 cells can transcriptionally activate the telomeric 1.A VSG gene by either the ELC or NDA mechanisms. While both mechanisms are used for the expression of different VSG gene families in a single serodeme^{16,18} and related VSG genes in different serodemes²², the present report shows that both mechanisms can be used for the expression of a single VSG gene within one serodeme.

Restriction maps of the 1.A VSG genes were constructed by genomic Southern analyses (Fig. 4). Each copy of the 1.A gene has an identical restriction map spanning the coding region from the 5' *SalI* site to the 3' *AccI* site. Outside this region the

Fig. 2 Southern blot analysis of the 1.A genes in early VATs and cloned relapses. DNA isolated from VATs IsTat 1.X^A, 1.Ae, 1.1^A, 1.Db, 1.A¹, 1.3a, 1.A³, 1.5a, 1.A⁵, 1.7a, 1.A⁷, 1.11a and 1.A¹¹ (see text for VAT nomenclature) was digested with restriction endonucleases *Sal*I (a) or *Hinc*II (b) and size-fractionated through 0.6% agarose gels. DNA isolated from VATs 1.Db digested with *Sal*I and 1.A¹ and 1.A⁵ digested with *Hinc*II did not digest to completion and the small internal and 5' *Hinc*II fragments ran off the gel in b. The gels were blotted bi-directionally onto nitrocellulose filters³⁵⁻³⁷, then hybridized with a 1.A cDNA clone (pTb1.A-2) labelled with ³²P by nick-translation³⁸. Size markers were *Hind*III, *Xba*I and *Kpn*I digests of λ DNA and their positions are indicated next to each autoradiogram. c, Densitometric scans of autoradiograms from *Hinc*II digests b normalized to give the same peak heights for the 2.0-kb band in each of the DNA digests. d, Restriction map of clones isolated from a cDNA library prepared from IsTat 1.A (refs 15, 16). Abbreviations used for restriction endonuclease sites are: A, *Ava*I; Ac, *Acc*I; B, *Bam*HI; Be, *Bst*EII; Bg, *Bgl*I; Bl, *Bgl*II; E, *Eco*RI; Hc, *Hinc*II; Hd, *Hind*III; Hf, *Hinf*I; P, *Pst*I; Pv, *Pvu*I; Pu, *Pvu*II; R, *Rsa*I; S, *Sal*I; Sp, *Sph*I; Ss, *Sst*I; Xh, *Xho*I.

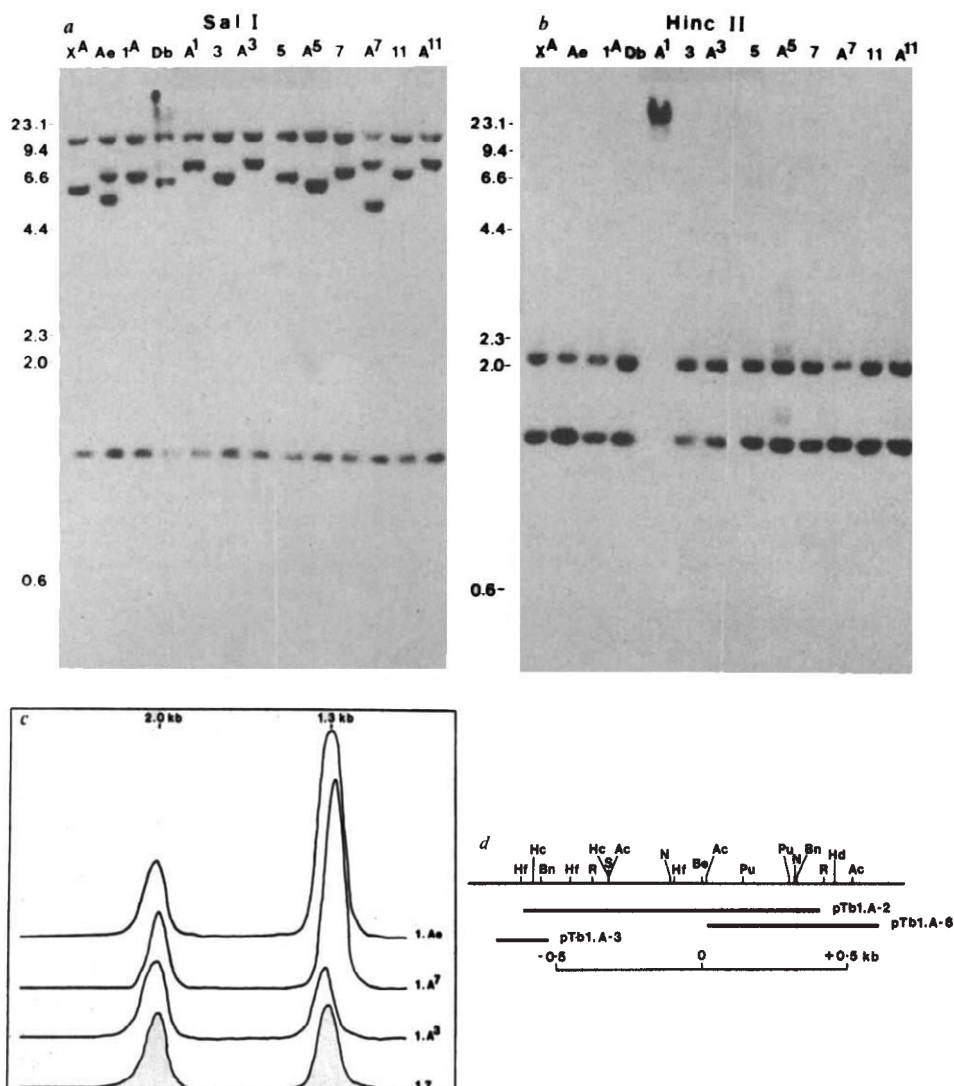
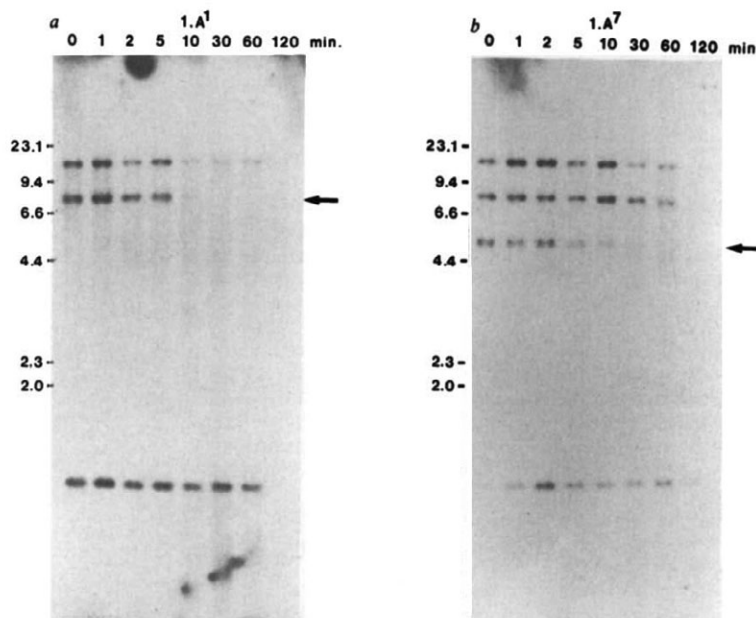


Fig. 3 DNase I sensitivity of IsTat 1.A genes. Frozen trypanosome pellets from 1.A¹ (a) and 1.A⁷ (b) were solubilized with Nonidet-P40 and treated with DNase I using the methods of Pays *et al.*¹². DNase I digested aliquots from the times indicated were deproteinized, digested with *Sal*I, electrophoresed in a 0.7% agarose gel and then blotted onto nitrocellulose. The filter was then hybridized with the 1.A probe (pTb1.A-2). The preferentially digested fragment from the expressed gene copy is indicated by an arrow.



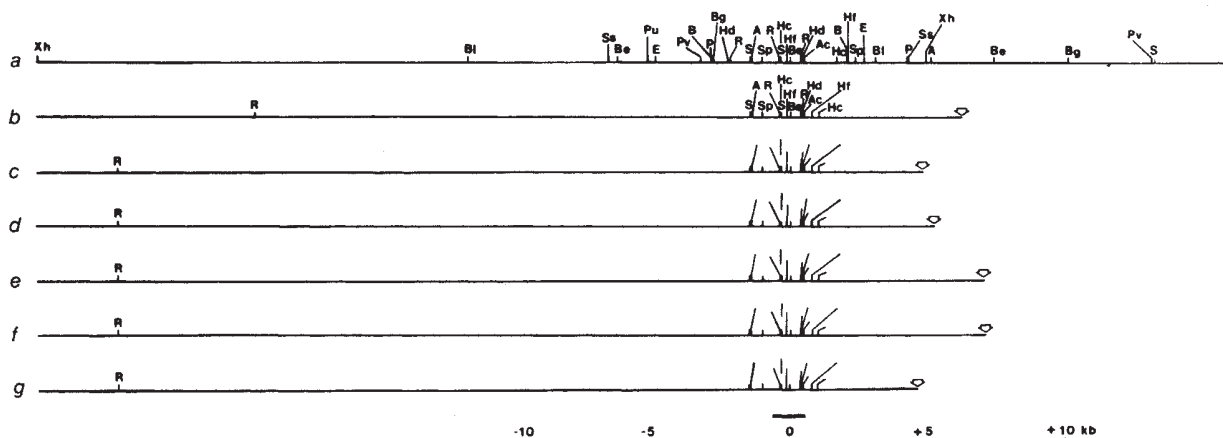


Fig. 4 Restriction maps of IsTat 1.A genes. *a*, The non-telomeric gene in all VATs. *b*, *c*, The ELC and telomeric BC genes, respectively, in IsTat 1.Ae. *d*, *e*, The telomeric gene in IsTat 1.Db and IsTat 1.A¹. *f*, *g*, The telomeric BC and ELC, respectively, in 1.A⁷. Restriction cleavage sites were determined by Southern hybridization analyses of genomic DNA (see, for example, Fig. 2). Several sites within the coding region have been omitted for clarity (see Fig. 2*d*). The abbreviations for restriction endonucleases used are shown in Fig. 2. The arrow denotes a 'universal restriction site' (*SalI*, *AvaI*, *SphI* and *BstEII*), indicating the telomeric end of a chromosome. The line above the scale indicates the 1.A VSG coding region.

non-telomeric gene (Fig. 4*a*) and telomeric genes (Fig. 4*b–g*) have different restriction maps. Except for the length of the telomere, the maps of the telomeric BC gene (Fig. 4*c–f*) are identical in all clones, whether or not the gene is expressed. Similarly, the map of the 1.A⁷ ELC (Fig. 4*g*) is identical to that of the telomeric 1.A⁷ BC (Fig. 4*f*). The 1.Ae ELC map, however, differs from these (Fig. 4*b*). The 5' 'barren' region is 5 kb shorter and the relative order of restriction sites 5' to the *RsaI* site distinguishes these telomeric expression sites (P.M., data not shown). The use of more than one expression site has also been seen with other ELCs in the IsTaR 1 serodeme²³ and in the BoTaR serodeme of *Trypanosoma equiperdum*²⁴, but is in contrast to previous results in *T. brucei*^{2,25}. To characterize these expression sites further, blots of restriction digests producing large (up to 50 kb) segments of 1.A 5' flanking sequence were hybridized with a synthetic probe complementary to a portion of the 35-nucleotide spliced leader found on the 5' termini of all VSG mRNAs^{26,27}. Although the probe hybridized with the large tandem array of directly repeated 1.4-kb units^{28,29} previously suggested to be the VSG gene expression site promoter²⁹, and with orphans^{28,30}, it did not hybridize to the large fragments containing the 1.A gene. Therefore, none of these spliced leader encoding sequences is within 50 kb of either the expressed or non-expressed 1.A genes.

Both non-telomeric^{12,31,32} and telomeric^{24,25,33,34} BC genes can be duplicated to form ELCs. The ELCs in both 1.Ae and 1.A⁷ are duplicated from the telomeric gene, since all their restriction maps are identical 3' to the 'barren' region and different from the map of the non-telomeric gene, especially the 3' *HincII* and *HinfI* sites (Fig. 4). The duplicated segment extends minimally from the 5' *SalI* site to the 3' *HincII* site and maximally from the 5' *RsaI* site to the end of the chromosome (Fig. 4). In 1.Ae, the 5' duplication boundary is between the 5' *SalI* and *RsaI* sites. Because the 5' maps of the ELC and BC genes in 1.A⁷ are identical, the boundary in this case may be further upstream. The 3' duplication boundary in both 1.Ae and 1.A⁷ must lie 3' to the *HincII* site. Thus, the ELCs in 1.Ae and 1.A⁷ may not result from duplication/transposition of a small 3–5-kb BC 'cassette' spanning the VSG coding region^{12,26,27}, but rather from a gene conversion initiated 5' to the coding region and extending to the end of the chromosome. Such a model is consistent with the results presented here and by others^{25,34}.

We have described a predominant VAT whose VSG gene can be expressed by both the NDA and ELC mechanisms. The fact that the telomeric 1.A gene can be activated by both mechanisms presumably allows its more frequent expression. Furthermore, the similarity between the genomic environments of the basic

copy gene and the 'expression site(s)' as shown here and elsewhere²⁵, may increase the probability of recombination between these two sites. The predominance of 1.A may thus be due to its ability to use alternative mechanisms for VSG gene activation, coupled with a higher frequency of recombination/gene conversion.

This work was supported by NIH AI17375, DAMD17-92-2016, WHO and Murdock Charitable Trust grants to K. S. and NIH AI17309 grant to N. A. and Rockefeller Foundation Support. We thank Elke Gobrecht for technical assistant and Dr Marilyn Parsons for useful discussions and critical reading of the manuscript.

Received 19 October 1983; accepted 19 March 1984.

1. Cross, G. A. M., Holder, A. A., Allen, G. & Boothroyd, J. C. *Am. J. trop. Med. Hyg.* **29**, 1027–1032 (1980).
2. Borst, P. & Cross, G. A. M. *Cell* **29**, 676–679 (1982).
3. Marcu, K. B. & Williams, R. O. in *Genetic Engineering* Vol. 3 (eds Setlow, J. K. & Hollander, A.) T129–T155 (Plenum, New York, 1981).
4. Gray, A. R. *Ann. trop. Med. Parasit.* **56**, 4–13 (1962).
5. Van Meirvenne, N., Janssens, P. G. & Magnus, E. *Ann. Soc. Belg. Med. trop.* **55**, 1–23 (1975).
6. Capbern, A., Giroud, C., Baltz, T. & Mattern, P. *Expl. Parasit.* **42**, 6–13 (1977).
7. Miller, E. N. & Turner, M. J. *Parasitology* **82**, 63–80 (1981).
8. Hajduk, S. L. & Vickerman, K. *Parasitology* **83**, 609–621 (1981).
9. Hoeijmakers, J. H. J., Frasch, A. C. C., Bernards, A., Borst, P. & Cross, G. A. M. *Nature* **284**, 78–80 (1980).
10. Agabian, N., Thomashow, L., Milhausen, M. & Stuart, K. *Am. J. trop. Med. Hyg. Suppl.* **29**, 1043–1049 (1981).
11. Pays, E., Van Meirvenne, N., Le Ray, D. & Steinert, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2673–2677 (1981).
12. Pays, E., Lhèreux, M. & Steinert, M. *Nature* **292**, 265–267 (1981).
13. Bernards, A. *et al. Cell* **27**, 497–505 (1981).
14. De Lange, T. & Borst, P. *Nature* **299**, 451–453 (1982).
15. Milhausen, M. *et al. Molec. Biochem. Parasit.* **9**, 241–254 (1983).
16. Parsons, M. *et al. Molec. Biochem. Parasit.* **9**, 255–269 (1983).
17. Williams, R. O., Young, J. R. & Majiwa, P. A. O. *Nature* **282**, 847–849 (1979).
18. Majiwa, P. A. O., Young, J. R., Englund, P. T., Shapiro, S. Z. & Williams, R. O. *Nature* **297**, 514–516 (1982).
19. Young, J. R., Donelson, J. E., Majiwa, P. A. O., Shapiro, S. Z. & Williams, R. O. *Nucleic Acids Res.* **10**, 803–819 (1982).
20. Stuart, K. *et al. J. Parasit.* (in the press).
21. Borst, P. in *Mobile Genetic Elements* (ed. Shapiro, J. A.) 619–656 (Academic, New York, 1983).
22. Miller, E. N., Williams, R. O. & Turner, M. J. *Nature* **306**, 196–198 (1983).
23. Myler, P., Allison, J., Agabian, N. & Stuart, K. *Cell* (submitted).
24. Longacre, S. *et al. Molec. cell Biol.* **3**, 399–409 (1983).
25. Laurent, M. *et al. Nature* **302**, 263–266 (1983).
26. Boothroyd, J. C. & Cross, G. A. M. *Gene* **20**, 281–289 (1982).
27. Van der Ploeg, L. H. T. *et al. Nucleic Acids Res.* **10**, 3591–3604 (1982).
28. Nelson, R. G. *et al. Cell* **34**, 901–909 (1983).
29. DeLange, T. *et al. Cell* **34**, 891–900 (1983).
30. Parsons, M., Nelson, R. G., Stuart, K. & Agabian, N. *Proc. natn. Acad. Sci. U.S.A.* **81**, 684–688 (1984).
31. Van der Ploeg, L. H. T., Bernards, A., Rijsewijk, F. A. M. & Borst, P. *Nucleic Acids Res.* **10**, 593–609 (1982).
32. Pays, E., Lhèreux, M. & Steinert, M. *Nucleic Acids Res.* **10**, 3149–3163 (1982).
33. Raibaud, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 4306–4310 (1983).
34. Pays, E. *et al. Cell* **35**, 721–731 (1983).
35. Southern, E. M. *J. molec. Biol.* **98**, 503–517 (1975).
36. Smith, G. E. & Summers, M. D. *Analyt. Biochem.* **109**, 123–129 (1980).
37. Wahl, G. M., Stern, M. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3683–3687 (1979).
38. Rigby, P. W. J., Dickermann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237–251 (1977).

DNA variation and evolution

FERRIS, Sage and Wilson¹ assayed wild-caught and inbred strains of mice (*Mus domesticus*) for variation in mitochondrial DNA (mtDNA) using restriction enzyme digestion. They concluded that the data showed either a common origin of 'old' inbred strains (probably a single female) or contamination from a single strain because all the 'old' inbred strains appeared to share a common mtDNA pattern found rarely (2 mice in 56 tested) in their survey of wild populations.

Their observations were made on samples of single mice from each of 14 inbred laboratory strains (5 'new' and 9 'old') and 15 wild populations; further samples of two and three mice were from two different origins. Thirty-six additional wild mice (origin not stated) were assayed using two restriction enzymes which could discriminate the 'old-inbred' pattern. When all members of a sample of size N are identical for a particular pattern, the 95% confidence intervals on the frequency of that pattern are: for $N = 1$, 0.025–1.00; for $N = 2$, 0.158–1.00; for $N = 3$, 0.292–1.00. Because the size of samples used in these experiments was small, it is possible that substantial levels of variation could remain undetected within the wild populations. Whilst the data may suggest a common origin of the 'old' inbred strains, the lack of data on variation within the wild populations precludes the elimination of the alternative hypothesis of independent origin.

In another paper, Coen, Thoday and Dover² based their estimation of rates of 'turnover' in structural variants of the ribosomal gene of *Drosophila melanogaster* on samples of two X chromosomes extracted from 10 pairs of iso-female lines and samples of 'at least two (usually three) X chromosomes from population cages established with a mixture of all iso-female lines. The authors assumed that if a variant was present in at least two sublines, it was probably derived from the founding population. On this basis no novel variants were detected in the paired iso-female lines and the rate of 'turnover' was estimated by assuming that no novel variants had been identified. However, no allowance was made for either the size of the samples or the sizes of the populations from which the samples were extracted. The maximum frequency at which novel variants could exist in all sublines and yet remain undetected ($P = 0.05$) in samples of 2 from 20 sublines is ~ 0.07 . In other words, the iso-female lines could be segregating for novel variants at fairly high frequencies and these would not be detectable because of the small sample sizes.

If these putative variants have arisen since the establishment of the lines, then the maximum rate of their production (m , the mutation or 'turnover' rate) is about 3.74×10^{-4} per generation which is of the same order as values obtained by Coen *et al.*² However, these estimates assume that the populations are large and no allowance is made for the finite size of the subline populations. Downes³ gives estimates of the effective population sizes (N_e) of the OK iso-female lines of between 90 and 400. At these values of N_e and m , the effects of the random sampling of gametes in each generation could have a considerable effect on the fate of new variants because the majority will be lost in the first generation. Consequently, the chances of detecting 'turnover' would be low in such small populations and the measurement of its rate made more difficult by the small sample sizes. Similarly the small samples taken from the cage populations and absence of any information on the effective population sizes restrict the interpretation of the data. The results quoted by Coen *et al.* provide no reasonable *prima facie* evidence of systematic change in the frequency of rDNA structural variants. The estimates of 'turnover' rates are based on the assumption that 'turnover' is occurring, not on a demonstration of its existence.

The reports of both Ferris *et al.*¹ and Coen *et al.*² describe the application of the techniques of molecular biology to investigate evolution. The procedures have exposed a range of variation which evolutionary biologists must consider seriously. However, the fascinating interpretations presented in these papers are not strongly supported by the data because alternative hypotheses cannot be eliminated. Acceptance of the conclusions of the two reports must await the availability of more data.

JOHN A. BARRETT

Department of Genetics,
University of Liverpool,
PO Box 147,
Liverpool L69 3BX, UK

1. Ferris, S. D., Sage, R. D. & Wilson, A. C. *Nature* **295**, 163–165 (1982).
2. Coen, E. S., Thoday, J. M. & Dover, G. A. *Nature* **295**, 564–568 (1982).
3. Downes, S. P. thesis, Univ. Cambridge (1981).

FERRIS *ET AL.* REPLY—'Concluded' is too strong a term to apply to the various possible explanations we offered for the remarkable finding¹ that all nine of the 'old inbred' strains of laboratory mice tested share a type of mtDNA whose

frequency is low (0.04) in wild mice (*Mus domesticus*). This finding contrasts sharply with published protein-electrophoretic evidence indicating that the nuclear genes of these laboratory strains are about as different from one another as are randomly picked nuclear alleles in wild mice¹. It was important not only to bring our finding to the attention of a wide audience but also to consider how to explain this puzzling contrast in light of the evidence that the mode of inheritance of mtDNA molecules differs from that of nuclear genes in being maternal.

Since these nine strains seen from published breeding records to be founded from at least five non-laboratory females, we calculated the probability of picking five mice with the 'old inbred' type at random from the wild. By 'at random from the wild', we meant at random geographically within the entire range occupied by wild members of the species *M. domesticus*. The best estimate of this probability, based on the frequency observed in our wild survey, is $(0.04)^5$, that is, 10^{-7} . This value, which has been confirmed by a more extensive survey², was so low that it led us to ask whether: (1) the five founding females were picked nonrandomly; (2) there was selection for the old inbred type; (3) there was frequent, unrecorded, contamination of one laboratory strain by another's mtDNA.

It was our position¹, which our subsequent work on many additional mice confirms^{2–4}, that none of these possible explanations for the mtDNA uniformity of the old inbred strains can yet be ruled out.

With regard to nonrandom sampling, we pointed to published evidence that most or all of the founding females came from the pet mouse trade. Drift or selection within this population could have brought about a high frequency of the 'old inbred' type of mtDNA. Alternatively, most of the wild mice used to establish or maintain the pet mouse population may have been trapped in a geographically nonrandom way, that is, from a particular region in which the 'old inbred' type happened to be at high frequency. Our initial survey of mtDNA in wild mice was a wide-ranging one, designed to detect all the major mtDNA lineages in *M. domesticus*¹. We were aware that until a much more extensive survey of mtDNA variability within and between wild populations of *M. domesticus* was conducted, this alternative could not be evaluated. Subsequent survey work^{2–4} bringing the total number of wild *domesticus* mtDNAs tested to 145, has confirmed our initial estimate of the frequency of the old inbred type; it has also shown, as we anticipated, that while there is heterogeneity within and between some localities as regards

mtDNA variability in *M. domesticus*, this species displays a rather weak tendency for macrogeographical structuring of that variability⁷.

S. D. FERRIS
R. D. SAGE
A. C. WILSON

University of California,
Department of Biochemistry,
Berkeley, California 94720, USA

1. Ferris, S. D., Sage, R. D. & Wilson, A. C. *Nature* **295**, 163–165 (1982).
2. Ferris, S. D., Sage, R. D., Prager, E. M., Ritte, U. & Wilson, A. C. *Genetics* **105**, 681–721 (1983).
3. Ferris, S. D. et al. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2290–2294 (1983).
4. Ferris, S. D., Ritte, U., Fischer Lindahl, K., Prager, E. M. & Wilson, A. C. *Nucleic Acids Res.* **11**, 2917–2926 (1983).

COEN ET AL. REPLY—Genetic turnover by unequal exchange and its evolutionary consequence of molecular drive is well established in the rDNA multigene family of diverse species, since the classic studies of Don F. Brown and colleagues in 1972 (for recent reviews see refs 1–3). To measure the rate of turnover we monitored, over several hundred generations, the stability of rDNA length variants, the expected products of unequal exchange^{4,5}, in isofemale lines of *D. melanogaster*⁶. Our experimental design was to compare individual X or Y chromosome rDNA variants taken from within each of several pairs of sublines, which had been established by splitting the F₁ progeny of each isofemale line and maintained separately over the prescribed time. By this means the stability of individual chromosome lineages could be assessed. Given that many X and Y chromosome variants from within subline pairs were shown to be identical (that is, no change was observed within these specific chromosome lineages) we estimated the maximal rate of unequal exchange based on the proper statistic that takes into account all independent unchanged lineages across all subline pairs. If the turnover rate were higher than this maximum, we would expect a smaller number of unchanged lineages than was actually observed. We note that Barrett concludes his ‘criticism’ with a paraphrase of our own summary that in the time course of the experiment “there is no *prima facie* evidence of systematic change in the frequency of rDNA structural variants”.

Barrett’s appeal for a larger sample size from each subline pair reveals his failure to understand our experimental design and underlines the dangers of applying statistics and population theory in the blind. Our estimated maximal rate is valid irrespective of the number of chromosomes assayed within each lineage and of problems of drift in small populations: it is based only on the number of individual

chromosome lineages, each assayed and considered independently. Hence the statistical estimate of our maximal rate would be improved by a wider sampling of more subline pairs, that is, more lineages, rather than more chromosomes within subline pairs.

E. S. COEN
J. M. THODAY
G. A. DOVER

Department of Genetics,
University of Cambridge,
Downing Street,
Cambridge CB2 3EH, UK

1. Dover, G. A. *Nature* **299**, 111–119 (1982).
2. Arnheim, N. In *Evolution of Genes & Proteins* (eds Nei, Coen, E. S., Strachan, T. & Dover, G. A. *J. molec. Biol.* **158**, 17–35 (1982).
3. Coen, E. S. & Dover, G. A. *Cell* **33**, 849–855 (1983).
4. Coen, E. S., Thoday, J. M. & Dover, G. A. *Nature* **295**, 564–568 (1982).

100 Hz is not the upper limit of synchronous muscle contraction

SMITH¹ has proposed that 100 Hz is the upper limit of contraction frequency in synchronous muscles—those muscles in which there is a 1:1 correspondence between motor neurone impulses and muscle contractions. In asynchronous muscles, contraction frequency can be

much higher than that of the motor neurone impulses. Smith reached his conclusion after demonstrating that whitefly flight muscles with high operating frequency are asynchronous rather than synchronous as was previously thought². 100 Hz may represent an upper limit of wing stroke frequency for synchronous flight muscles, but several synchronous muscles have significantly higher operating frequencies, notably sound-producing muscles in bats, fish, crustacea and insects^{3–9}.

We have studied cicada tymbal muscles, which contract at high frequency during sound production. These muscles are especially pertinent because a wide range of contraction frequencies is found by examining different species¹⁰ and because both synchronous and asynchronous tymbal muscles occur¹¹. Our physiological and fine-structural data show that some synchronous tymbal muscles operate at over 200 Hz (224 Hz in *Psaltoda claripennis*).

In synchronous muscles, high contraction frequencies are made possible by muscle modifications that result in short twitch duration. A comparison of several species of cicadas having synchronous muscles¹⁰ shows a close correlation between twitch duration and contraction frequency (Fig. 1a) which extends over the full range of contraction frequencies. However, one species, *Platypleura capitata*, which has asynchronous muscles¹¹, is vastly different in that it has a very short cycle period during singing but a relatively long twitch duration. This is to be expected as high operating frequencies

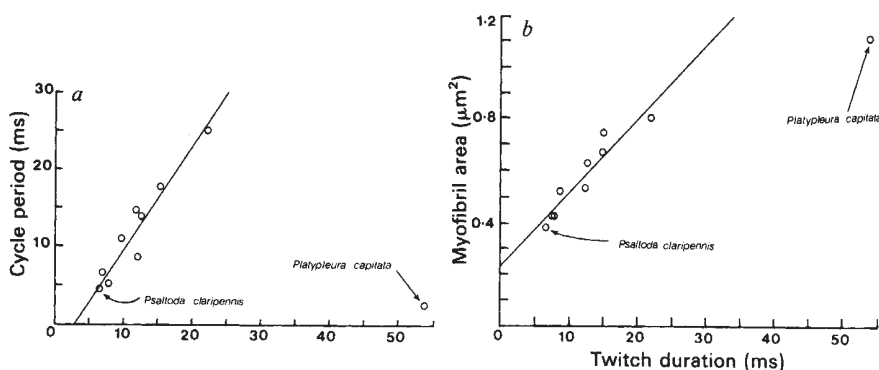


Fig. 1 a, The inferred cycle period (=reciprocal of contraction frequency) during the calling song compared with the isometric twitch duration (onset to 50% relaxation, at 30 °C) of the partially isolated tymbal muscles for 10 species of cicada. b, Comparison of the myofibril area (measured stereologically from electronmicrographs of transverse sections) with the isometric twitch duration in tymbal muscles for 10 species of cicada. In a and b, all the species have synchronous tymbal muscles except for *Platypleura capitata*, which is asynchronous. The lines are least-squares regression lines for the data, excluding *P. capitata* ($r = 0.95$ in a and b). In a, the unlabelled points represent the following species, in order of increasing twitch duration: *Psaltoda harrisii*, *Psaltoda argentata*, *Magicalicada cassini*, *Magicalicada septendecim*, *Cyclochila australasiae*, *Abricta curvica*, *Chlorocysta viridis* and *Cystosoma saundersii*. Details of these species are presented elsewhere^{10,11}. Two species (*Arunta perulata* and *Tamasa tristigma*), in which there is some doubt about the cycle period during calling, are not included in this graph. In b, the unlabelled points represent the following species, in order of increasing twitch duration: *P. harrisii*, *P. argentata*, *T. tristigma*, *C. australasiae*, *A. curvica*, *A. perulata*, *C. viridis* and *C. saundersii*. Details of tymbal muscle fine structure for these species will be published elsewhere (R.K.J. and D.Y., in preparation). Data for *P. capitata* are given in ref. 11. *M. cassini* and *M. septendecim* were not included in the study of fine structure.

in asynchronous muscles are achieved by elaboration of stretch activation/release inactivation in muscles that basically have twitches of long duration^{12,13}.

Synchronous and asynchronous muscles can be distinguished by their fine structure, as Smith¹ points out; the former possess an extensive sarcoplasmic reticulum and T-system and tend to have small myofibrils, whereas the latter tend to have a very reduced sarcoplasmic reticulum and large myofibrils. In cicadas, twitch duration in synchronous tymbal muscles is inversely related to the quantity of sarcoplasmic reticulum within each muscle fibre (R.K.J. and D.Y., in preparation). The faster tymbal muscles also have smaller myofibrils, with myofibril cross-sectional area being the best morphological predictor of twitch duration (Fig. 1b). The asynchronous muscle of *P. capitata* is quite different from fast, synchronous muscle (for example, that of *P. claripennis*) in having a large fibril area and in being sparsely supplied with sarcoplasmic reticulum¹¹.

Thus, synchronous tymbal muscles constitute a graded series, throughout which contraction kinetics and fine structure are closely correlated with the normal operating frequency. We have demonstrated that contraction frequencies of over 200 Hz are achieved within the synchronous mode of operation. Clearly, 100 Hz is not the upper limit of contraction frequency in synchronous muscles.

DAVID YOUNG

Department of Zoology,
University of Melbourne,
Parkville, Victoria 3052, Australia

ROBERT K. JOSEPHSON

Department of Psychobiology,
University of California,
Irvine, California 92717, USA

1. Smith, D. S. *Nature* **303**, 539–540 (1983).
2. Wootton, R. J. & Newman, D. J. S. *Nature* **280**, 402–403 (1979).
3. Revel, J. P. *J. Cell. Biol.* **12**, 571–588 (1962).
4. Suthers, R. A. & Fattu, J. M. *Am. Zool.* **13**, 1215–1226 (1973).
5. Skoglund, C. F. J. *Biophys. biochem. Cytol.* **10** (Suppl.), 187–200 (1961).
6. Cohen, M. J. & Winn, H. E. *J. exp. Zool.* **165**, 335–370 (1967).
7. Mendelson, M. J. *Cell Biol.* **42**, 548–563 (1969).
8. Josephson, R. K. & Halverson, R. C. *Biol. Bull.* **141**, 411–433 (1971).
9. Josephson, R. K. *J. exp. Biol.* **59**, 781–801 (1973).
10. Young, D. & Josephson, R. K. *J. comp. Physiol.* **152**, 183–195 (1983).
11. Josephson, R. K. & Young, D. *J. exp. Biol.* **91**, 219–237 (1981).
12. Pringle, J. W. S. *Proc. R. Soc. B* **201**, 107–130 (1979).
13. Pringle, J. W. S. *J. exp. Biol.* **94**, 1–14 (1981).

Encephalization in *Proconsul africanus*

WALKER *et al.*¹ recently suggested that the Miocene hominoid *Proconsul africanus* was more encephalized than extant monkeys of similar body size, and they suspected that it may resemble pongids in relative brain size. As an index of encephalization

Table 1 Encephalization indices in *Proconsul africanus* and extant apes and monkeys

	EQ	CC	N _c (×10 ⁹ neurones)
<i>Proconsul africanus</i>	48.8	19.6	1.9
<i>Pan troglodytes</i>	41.1	35.6	3.6
<i>Pongo pygmaeus</i>	31.6	30.1	3.3
<i>Gorilla gorilla</i>	17.2	31.2	3.5
Cercopithecoidea	22.9–82.0	8.0–20.2	0.9–2.1

EQ = brain weight (g)/0.0991 (body weight, g)^{0.76237}; CC = brain weight (g)/(body weight, g)^{0.23}; for calculation of N_c, see ref. 6.

they used an encephalization quotient (EQ) based on a particular allometric equation². Here I re-examine the degree of encephalization of *P. africanus* by applying two additional, but different, indices of encephalization traditionally used in brain–body size studies: one based on the constant of cephalization (CC)^{3–5}, the other on the extra neurone index (N_c)⁶. Both approaches yield the same result, which is in conflict with that of Walker *et al.* According to these methods, the degree of encephalization of *P. africanus* is within the range of extant monkeys and substantially below that of the apes.

The equation used by Walker *et al.* provides an excellent fit for brain–body size relationships in high-level taxa such as the class Mammalia (mouse–elephant curve) or the order Primates (mouse lemur–gorilla curve). Because of its high exponent of allometry (0.76), however, it tends to overestimate the degree of encephalization for small-sized species while underestimating encephalization in larger species, when comparisons are made at a lower level of the hierarchy. This is the case because among closely related species or genera, brain weight scales at a much lower power of body weight with values of the exponent of allometry characteristically falling between 0.2 and 0.3 (refs 3, 4, 7, 8). In the study by Walker *et al.* this phenomenon leads to obviously erroneous results such as gorillas being less encephalized than the least encephalized monkey and the range for monkeys surpassing that of the apes at the upper end by a considerable margin (Table 1). Being aware of the strong limitations of their method, Walker *et al.* resort to a comparison of *P. africanus* with similar sized monkeys. While this alleviates the problem somewhat, the fact that the comparison of EQs is based on an equation fitting high-level taxa still leaves a major source of error.

Using the same brain–body size data but an equation based on an exponent of allometry (0.23) consistent with brain–body size relationships among low-level taxa, yields not only different but much more consistent results (Table 1). The CC values for the apes are closely grouped together and substantially above the range for monkeys. *P. africanus* lies towards the upper end of the monkey range. For

P. africanus to be at the bottom of the range of apes (CC = 30) would require a cranial capacity of 255 cm³, almost 90 cm³ larger than the actual estimate, at a body weight estimate of 11 kg. Use of the extra neurone index yields the same results (Table 1): apes fall closely together and are clearly above the monkey range with *P. africanus* in the upper end of the latter.

Thus, while *P. africanus* may share several derived traits with extant apes, relative brain size does not seem to be one of them.

WALTER LEUTENEGGER

Department of Anthropology,
University of Wisconsin,
Madison, Wisconsin 53706, USA

1. Walker, A., Falk, D., Smith, R. & Pickford, M. *Nature* **305**, 525–527 (1983).
2. Holloway, R. L. & Post, D. G. in *Primate Brain Evolution* (eds Armstrong, E. & Falk, D.) 57–76 (Plenum, New York, 1982).
3. Hemmer, H. *Proc. 3rd int. Congr. Primatol.* (eds Biegert, J. & Leutenegger, W.) 99–107 (Karger, Basel, 1971).
4. Leutenegger, W. *Folia Primatol.* **19**, 9–17 (1973).
5. McHenry, H. M. *Nature* **254**, 686–688 (1975).
6. Jerison, H. J. *Hum. Biol.* **35**, 263–291 (1963).
7. Röhrs, M. *Z. wiss. Zool.* **162**, 1–95 (1959).
8. Gould, S. J. *Contr. Primatol.* **5**, 244–292 (1975).

SMITH AND WALKER REPLY—Leutenegger's discussion and data are a useful addition to the interpretation of relative brain size in *Proconsul africanus*, but careful evaluation of his data confirms our conclusion that *P. africanus* shows a tendency towards the increased encephalization that characterizes apes in comparison with monkeys. The indices used by Leutenegger do not eliminate the confounding effect of size that he correctly noted exists for the values that we reported when using a slope of 0.76 to calculate encephalization quotients.

While our measurement of relative brain size involved an inverse bias—animals with larger body weights having lower EQs—both indices used by Leutenegger show the opposite bias—larger animals having larger values. He reports Hemmer's¹ values for the constant of cephalization (CC) as being in the range 8.0–20.2 for Cercopithecoidea. The value of 8.0 is for the smallest cercopithecoideid, *Miopithecus talapoin*, and the largest

Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 500 words and the briefest of replies, to the effect that a point is taken, should be considered.

values are for several of the largest species in the group, all from the genus *Papio*. With the extra neurone index (N_e), Jerison² reported that elephants have a value twice that of humans and porpoises have about the same values as we do. At the other end of the scale, the small South American squirrel monkey, *Saimiri sciureus*, was grouped with lemurs and marmosets rather than with more closely related, but larger, New World monkeys. Jerison himself noted that "If we demand that the values of N_e correspond to an ordering in terms of behavioural capacities we must assume either that the assumptions used in determining N_e are insufficient or that we grossly underestimate the behavioural capacities of the elephant and porpoise. I would guess that both types of error occur, but I would prefer, for the present, to emphasize the second."

If, as these results suggest, there is a tendency for CC and N_e to be underestimated for small species within a taxon, our suspicions of increased encephalization in *P. africanus* remain plausible. For both CC and N_e , the values for *P. africanus* listed by Leutenegger are close to the highest values for all monkeys and the monkeys with the higher values are also considerably larger than *P. africanus* in body size. *P. africanus* has lower values than the extant great apes, but also has a much smaller body size than these.

RICHARD SMITH

Department of Orthodontics,
University of Maryland
Dental School,
Baltimore, Maryland 21201, USA

ALAN WALKER

Department of Cell Biology
and Anatomy,
The Johns Hopkins University
School of Medicine,
Baltimore, Maryland 21205, USA

1. Hemmer, H. *Proc. 3rd. int. Congr. Primatol.* (eds Biegert, J. & Leutenegger, W.) 99-107 (Karger, Basel, 1971).
2. Jerison, H. *J. Hung. Biol.* 35, 263-291 (1963).

RECENTLY Toh *et al.*¹ reported the existence of amino acid sequence similarity between the reverse transcriptase of certain retroviruses and the DNA polymerases of cauliflower mosaic virus (CaMV) as well as a region of hepatitis B virus (HBV) that probably encodes a DNA polymerase. For completeness, we draw attention to other regions of protein similarity between retroviruses (human T-cell leukaemia virus, HTLV; Moloney murine leukaemia virus, Mo-MuLV; and Rous sarcoma virus, RSV), HBV and CaMV that were not reported by Toh *et al.*

The sequences are presented in Table 1 and the relative positions of the similar regions indicated in Fig. 1. The common set of amino acids discussed by Toh *et al.* is conserved in the amino-terminal region of all retrovirus reverse transcriptases studied (regions I-III). These common sequences are located near the amino-terminus of the HBV protein and the centre of the CaMV polymerase. Another region of similarity, IV, not discussed by Toh *et al.*, is also found in all retroviruses and is present in the CaMV protein. Region V, reported by Toh *et al.*, to be present in Mo-MuLV and RSV, is also found on HTLV. We also draw attention to sets of amino acids that are conserved

Fig. 1 Alignment of the polymerase gene products among five different viruses, depicting the regions shown in Table 1.

with respect to sequence and relative position between the reverse transcriptases of HTLV and Mo-MuLV (M1-M4) and HTLV and RSV (R1-R4).

ROBERTO PATARCA

WILLIAM A. HASELTINE

Laboratory of
Biochemical Pharmacology,
Dana Farber Cancer Institute,
Harvard Medical School,
Boston, Massachusetts 02115, USA

1. Toh, H., Hayashida, H. & Miyata, T. *Nature* 305, 827-829 (1983).
2. Shinnick, T. M., Lerner, R. A. & Sutcliffe, A. *Nature* 293, 543-548 (1981).
3. Seiki, M. & Hattori, N. *Proc. natn. Acad. Sci. U.S.A.* 80, 3618-3622 (1983).
4. Schwartz, E., Tizard, R. & Gilbert, W. *Cell* 32, 853-869 (1983).
5. Gardner, R. C. *et al. Nucleic Acids Res.* 9, 2871-2888 (1981).
6. Ono, Y. *et al. Nucleic Acids Res.* 11, 1747-1757 (1983).

Table 1 Alignment of the similar regions in polymerase gene products among different viruses

REGION	VIRUS	SEQUENCE	REGION	VIRUS	SEQUENCE
I	HTLV Mo-HuLV RSV CaMV	114 270 107 155 DLRDAFFQIP DLKDAFFCERL DLKDCFFSIP DCKSGFGVYLL	M1	HTLV Mo-HuLV	720 974 TDNGPAYIS TDNGPAEVS
II	HTLV Mo-HuLV RSV CaMV HBV	150 305 143 384 25 WKVLPQGFKNSTLF WTRLPQGFKNSTLF WKVLPQGFKNSTLF WVLPFGGLKQPSIF ERKIFMGVGLSPFL	M4	HTLV Mo-HuLV	745 799 HVPYHPTSSGLVERSN HCAVAPQSSGQVERMN
III	HTLV Mo-HuLV RSV CaMV HBV	184 338 177 173 37 ILQYMDILLASPSH ILLYYDDLLAATSE LLQYMDILLASSSH CCYYDDLLVFSHNE LAFSYMDYVLLGAKSH	R1	HTLV RSV	37 31 LQALQHLVRKALEAGHIEP LVALTQLVEKELSLGHIEP
			R2	HTLV RSV	61 55 NNPVFPVKKANGTWRFIHDLRAITN NTPVFVIRKASGSRLEHDLRAIVN
IV	CaMV Mo-HuLV HTLV RSV	331 727 541 556 DAYNLPNKDELLTIRGKKIFSSF EGKEIKNKDELLALLLKALFLPKRLLS LALGTFFGRSSQAPFQALLPLRLLS FFTEGNQVADSQAATFQAVYPLREACK	R3	HTLV RSV	572 546 HVRSH HVRSH
			R4	HTLV RSV	660 633 IWQGDIT IWQGDIT
V	HTLV Mo-HuLV RSV	672 931 650 LHVWYDTFSGAIIISA LLVEEDTFSGWIIIEA LAVVDTFSGAIIIVV			
M1	HTLV Mo-HuLV	263 149 QALL QALL			
M2	HTLV Mo-HuLV	799 673 PPHKSAQRAELLGL PAGTSAQRAELLAL			

Moloney murine leukaemia virus, Mo-MuLV²; human T-cell leukaemia virus, HTLV³; Rous sarcoma virus, RSV⁴; cauliflower mosaic virus, CaMV⁵; and hepatitis B virus, HBV⁶. Common amino acids are boxed and conservative substitutions with respect to HTLV are underlined. For each sequence, the first amino acid is numbered.

Primates by numbers

Bernard Wood

The Order of Man: A Biomathematical Anatomy of the Primates. By Charles Oxnard. Yale University Press: 1984. Pp.366. \$30, £20.

THE manner of acquiring knowledge about ourselves and our closest relatives, the primates, perceptibly changes every few years, so that the past two or three decades have witnessed the virtual transformation of primatology. Prior to this, apart from observations of captive animals and a few pioneering field studies, primatology was all but confined to classical comparative anatomical and palaeontological research. Primatology, or more accurately primate evolutionary biology, has now matured into a discipline which embraces studies as disparate as molecular evolution, ethology and comparative neurobiology.

Nonetheless, investigations of the form of the skeleton, and comparisons of homologous skeletal parts, still play a prominent part in primate studies. The advent of computers, along with statistical techniques which allow several variables to be considered simultaneously, have progressively added to the power and range of these so-called morphometric methods, and this book is the most recent of a trilogy* by Charles Oxnard which describes the basis, application, evolution and impact of the newer morphometric techniques. *The Order of Man* is particularly interesting because, more than either of the other books, it attempts to set these studies within the broader context of primatology.

Morphometry is the reduction of a complex form, usually a bone, to numbers. Present-day techniques are sophisticated in three respects. The first is the ingenuity of the ways in which shape and size are recorded: simple linear measurements are being superseded by three-dimensional co-ordinate and orthogonal analysis, as well as by optical techniques. The second is the application of morphometry to the internal structure of bone, thus increasing the comparative information which can be gleaned from skeletal material. Finally, there is the statistical treatment of the data. Multivariate techniques have been refined and elaborated so that the elements of a complex three-dimensional shape can be visualized and more easily compared with other homologous patterns.

In the United Kingdom, it was the Birmingham School of anatomists, led and inspired by the now Lord Zuckerman, who, in the post-War period, brought

bivariate, and then multivariate statistics to the study of the skeletons of living and fossil primates. Claims for the recognition of new and different sorts of hominids were often, and sometimes correctly, based on morphological features; but, with some honourable exceptions, little attempt was made to support these decisions with quantitative data. To those unfamiliar with the morphology of such material it was easy, but unfair, to interpret all this work as unsound. Nonetheless, even when data could be assessed metrically, Zuckerman and his statistical collaborators saw the flaw in methods which implied that each measurement provided a separate "proof" of difference or affinity. They pioneered the introduction and use of simple statistical tests, and later used multivariate methods which were able to identify, and allow for, correlation and covariance between measurements. This work was continued by E.H. Ashton and Oxnard, who represent the first and second generations of the Birmingham School. Though much of their work has been collaborative, Oxnard has emerged as the spokesman, and indeed the doyen, of primate morphometry. So this book doubles as a statement of personal scientific philosophy, as well as being a prospectus for the morphometric cause.

Morphometry has two main applications in primatology — the analysis of form in terms of, first, function and, second, affinity. As I understand it, the gist of the first application, as it has been applied to locomotion, is the following. Individual primate taxa show a range of locomotor and positional behaviours. Thus, the skeleton has to tolerate a range of imposed stresses and strains. These methods seek to find out whether some activities — leaping and hanging, for example — impose such constraints on skeletal variation (or vice versa) that the activity can be recognized in the bones. The second application, the essentially phenetic comparison of form, has found most use in the study of fossils, particularly fossil hominids. All fossils, like living individuals, are unique, but the problem is how to assess the significance of the differences between them. If they are greater than the variation in analogous modern taxa, or are qualitatively different from those seen in intraspecific variation, then taxonomic distinction is warranted. Morphometry provides the means to make these comparisons and assessments.

Most of *The Order of Man* is taken up with describing a series of case studies in which morphometry has been applied to

various regions of the skeleton. The detractors of multivariate methods will claim that what these studies have revealed is evident to an experienced anatomist, but this is altogether too glib an assessment. By untangling the web of intercorrelation, such methods reveal patterns of association otherwise concealed from conventional analysis, and some studies, particularly those on the scapula and the pelvis, have provided important functional insights. However, perhaps more interesting than the results themselves is the way that Oxnard regards them. He apparently no longer sees these techniques as part of the armamentarium of the primatologist, but instead regards morphometry as one of three, seemingly discrete, approaches which can be used to determine relationships among primates (the other two being classical morphology and molecular evidence). Oxnard appears to see these technique-based divisions as somehow being in competition, and he points to the concordance of patterns shown by the molecular and morphometric techniques as evidence of the efficacy of the latter.

Apart from the dubious logic of such tests, the examples of concordance which are quoted are not as reliable as is implied in the text. For example the apparent agreement between morphometric and molecular evidence, that *Tarsius* is the sister group of the anthropoids, is contrasted with the conclusions of classical studies which class it as a prosimian. However, the molecular evidence for *Tarsius* is at best equivocal, and there is cogent evidence from classical morphology to suggest that *Tarsius* should be included, with the anthropoidea, in the Haplorhini. These details apart, I am dismayed to see a holistic approach to primate studies abandoned in favour of a compartmentalized one. It neglects other important lines of evidence, behaviour for example, and it implies that the three broad approaches provide the same type of information. Although some multivariate techniques take account of within-group correlation, the raw material of morphometrics is phenetic data. While some — perhaps even most — of this data reflects patristic affinity, part of it is likely to reflect parallelism and convergence, and thus confound attempts to determine "true" relationships. This philosophical shift in the way Oxnard views the evidence of comparative morphometrics, towards regarding it as suitable grist for the primate systematic mill, is thus a major change. It is also one which runs counter to the emphasis in recent years, exemplified by cladistic methods, which stresses the importance of analysing the total form of an animal or a bone, to distinguish between characters which are shared with a wide range of taxa, and those which are unique to a set of taxa, or a single taxon.

The last part of the book concentrates almost exclusively on assessing affinities,

**Form and Pattern in Human Evolution* (1973); *Uniqueness and Diversity in Human Evolution: Morphometric Studies of Australopithecines* (1975). Both published by The University of Chicago Press.

particularly those of hominid fossils. Along with many other workers, Oxnard and his colleagues have used morphometric and other evidence to demonstrate convincingly that the australopithecines are different from both modern man and the extant apes; in short, that they are unique. This generalization has been accepted by most hominid palaeontologists, even though some specimens such as the foot (OH 8) from Olduvai are still the object of the palaeontological equivalent of hand-to-hand fighting. Oxnard is right to claim credit for contributing to this conclusion. But he is wrong to suggest that it was morphometric evidence alone which brought about the change.

There is, however, less agreement about the significance of this phenetic uniqueness. While Oxnard and his colleagues regard it as debarring both the robust and gracile australopithecines from an ancestral relationship to *Homo*, others see one, or both, of the gracile australopithecine taxa as potential ancestors of either the *Homo* or robust australopithecine clades, or perhaps both. The former position would be vindicated if a more suitable *Homo* ancestor were to be found which was either synchronic with, or older than, the australopithecines. Oxnard makes this prediction explicit in the book, so it is particularly puzzling that he includes a *nota bene* section which draws attention to the generally "ape-like" morphology of *Australopithecus afarensis*. If anything, current interpretations of this earlier form of gracile australopithecine tend to emphasize the relatively *Homo*-like features of *A. africanus*, and it is by no means certain that, despite its "morphometric" uniqueness, it can be excluded as a common ancestor of both *Homo* and the robust australopithecines. It is simply not true, nor has it been for a decade or more, to say that patterns of human evolution in the literature "all conform to the notion of a single lineage". Since the abandonment of the "single-species" hypothesis most, if not all, hominid phylogenetic trees have incorporated a separate robust australopithecine lineage, and the recent schemes of Johanson, White and Kimbel incorporate the gracile australopithecines within it. Thus, in continuing to attack the

unilinear evolutionary model, Oxnard spends most of the concluding chapter delivering a *coup de grace* to an opponent that has long since left the field. Oxnard is not familiar with the fossil evidence, and it shows throughout this section.

The design of the book and style of the text deserve comment, for they reflect Charles Oxnard's laudable desire to innovate. The layout is pleasing to the eye, but at times infuriating for the reader. Many figures are three or four pages away from the relevant text, and one is eight pages adrift. Despite the desire expressed in the preface that *The Order of Man* should be accessible to the beginner and the layperson, and the inclusion of an abstract and a summary for each chapter, this is clearly not a book for the non-specialist. Nobody is better at explaining these difficult techniques than Oxnard, and indeed the early chapters discuss some general methodological problems with commendable conciseness and clarity. But most of the book is made up of highly technical diagrams, and the text takes for granted a considerable grasp of primate taxonomy and of systematics on the part of the reader.

The Order of Man is not merely a compilation of the published work of the descendants of the Birmingham School, yet neither is it an objective or comprehensive review of the contribution of morphometrics to primate studies. There is a dearth of self-criticism, and perhaps a surfeit of self-congratulation. It is as if the author were so impatient of the assessments of others, or so exasperated by the lukewarm response of his peers to published analyses, that he decided to act as his own judge and jury. Oxnard and his colleagues have offered much to current debates in primatology, but this text makes it no easier to understand where they are bound, apart from the prospect of mega-computations based on data from both limbs and trunk combined. I fancy that in the decades ahead this unusual book will be of as much interest to historians of science as it will to primatologists. □

Bernard Wood is Courtauld Professor of Anatomy at The Middlesex Hospital Medical School, University of London.

Vandalized space

Ian G. Halliday

Quarks, Gluons and Lattices.

By M. Creutz.

Cambridge University Press: 1983.

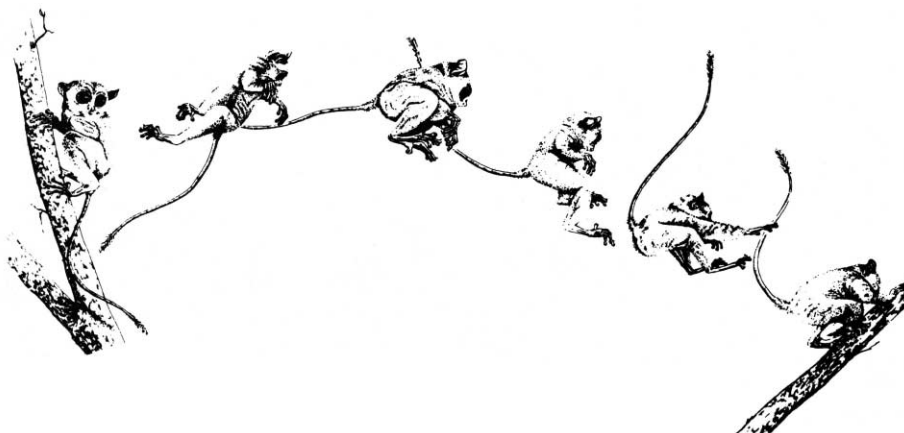
Pp. 169. £17.50, \$34.50.

RELATIVISTIC quantum field theory poses some of the hardest and most intriguing problems known to theoretical physicists. On the one hand the divergent perturbation series of quantum electrodynamics correctly predicts the magnetic moment of the electron to eleven significant places. On the other hand the experimentalists insist that protons are made of quarks, which refuse to behave as naive analogues of the proton and neutron in the nucleus or of the electron and proton in the hydrogen atom. No matter how hard one bangs protons together no quarks come out. Yet starting from a Hamiltonian written in terms of quark and spin coloured gluon fields, any application of perturbation theory would give free quarks.

So, theoretical physicists have been forced to alternative non-perturbative approximation schemes. The most popular is, at first sight, the most unlikely. Space-time is vandalized and reduced to a discrete, finite volume lattice. This kills all the sacred cows of translation invariance, Lorentz invariance and rotation invariance. We are off to a bad start. However, the system now consists of a finite number of degrees of freedom. Thus it is amenable to direct numerical simulation. Also, it is now very like a solid-state problem where much intuition has been accumulated. For example, near a second-order phase transition the correlation length or inverse mass goes to infinity and details of the lattice are washed out. This is the route to recovering Lorentz invariance! The limit must be taken in parameter space to a point where the correlation length becomes infinite in lattice spacings. This is equivalent to the lattice spacing tending to zero as required to recover the original field theory.

The purpose of Mike Creutz's elegant book is to provide a gentle introduction to the basic results in lattice field theory. It succeeds admirably. Without drowning the reader in detail, sufficient background is given to make the book very readable and provide an easy route to the research literature. Enough examples of calculations are given to develop real confidence in manipulating gauge groups, group integrals, duality and all the other tools of the trade. I recommend the book in the strongest terms to all interested in the fundamental structure of nature and the surprises quantum field theory has in store.

Ian G. Halliday is a Reader in Theoretical Physics at Imperial College, University of London.



Focus on fleas

Harry Hoogstraal

The Rothschild Collection of Fleas: The Ceratophyllidae.

By Robert Traub, Miriam Rothschild and John Haddow.

Published privately (distributed by Academic Press): 1983. Pp.494. £60, \$99.

WE have here a contradiction to the statement: "No great and enduring volume can ever be written on the fleas, though many there be who have tried it" (H. Melville in *Moby Dick*, Chapter 104). Preceded by five volumes of the *Illustrated Catalogue of Fleas (Siphonaptera) in the British Museum (Natural History)*, this review of the Ceratophyllidae has been particularly difficult and time-consuming to prepare. Since 1936, when the last critical review of the family was published, the number of component species has increased from 71 to about 470 in 65 genera and subgenera, or about 20% of all known fleas.

As in the previous volumes, the specimens in the Rothschild and British Museum collections are listed. However, because of the sheer number of ceratophyllid species, the format of this volume differs from that of the first five. The keys (by F.G.A.M. Smit) to genera and subgenera (seven genera and eight subgenera are described as new) are preceded by a detailed account of morphology and a table of comparative morphology of the closely related family Leptopsyllidae, and are illustrated by 205 line-drawings and 90 photomicrographs (by J. Navarro and R. Traub) of definitive structures of each genus.

The sections on zoogeography and distribution, and the notes on hosts, include comments on the ecology of each ceratophyllid species. A distribution map accompanies the introductory remarks for each genus. The 151 full-page maps show collecting localities of each species on a background of the distribution of the chief host — a formidable task for much of the world, when dealing with so many different flea and host species from so many remote localities.

The review of hosts of ceratophyllids is a classic exposition of host-parasite relationships, special reference being made to the historical distribution of the chief hosts (especially the more-recently evolved Sciuridae, Cricetidae, Geomyidae; and certain birds). It appears to me that the nesting types of many important avian hosts (woodpeckers, swallows, weaver birds, buntings and so on) of ceratophyllids may have a significant role in their infestations by these fleas. The reasoning ("negative evidence") for the dearth of ceratophyllids on Muridae, other rodents and mammals of other orders is as important for biologists to understand as the reasons advanced for why

ceratophyllids parasitize their chief mammalian hosts.

This section leads on to an equally valuable account of the evolution of the Ceratophyllidae from the standpoints of historical biogeography and host-relationships, morphology, taxonomy, phylogeny, physiology, behaviour and geological age. The conclusions are that these fleas, like their major and aboriginal hosts (Sciuridae), arose in the Nearctic Region about 30–40 million years ago (early Oligocene) and that they are also the most recently evolved and highly developed group of Siphonaptera.

In the chapter on medical importance, Traub stresses that ceratophyllids do not have a large role in the epidemiology of plague and murine typhus but are clearly important as vectors of zoonotic agents among small mammals, primarily rodents, which are the ultimate source of human disease. The introductory remarks on factors affecting epidemiology illustrate Traub's broad experience in the area of arthropod-borne diseases. Ceratophyllids are discussed in relation to plague, tulare-

mia, other bacterial infections, rickettsiae, viruses, and other infections and medical aspects.

Three appendices complete the book, the first listing species described since 1975, the second dealing with the orders of hosts (and the number of families, genera and species in each) and the number of ceratophyllids (genera/subgenera) and other flea families associated with these host orders and families. Finally, Appendix 3 is an alphabetical list of host genera together with the major ceratophyllid species infesting each.

In the preface, the authors state that their aim was to provide a sound base for future work on this family of parasites and to assist the medical entomologist in the task of identifying his specimens. They have amply fulfilled their mission, and have contributed much solid data and conceptual wisdom to the biomedical sciences. □

Harry Hoogstraal is Senior Scientist at the US Naval Medical Research Unit No.3, Cairo, Egypt, and President-elect of the American Society of Parasitologists.

Policy for science

Harvey Brooks

Science in Government: A Review of the Principles and Practice of Science Policy.

By J. Ronayne.

Edward Arnold: 1984. Pp.250.

Pbk £9.95, \$19.95.

FOR the reader familiar with recent writings on science policy, there are few surprises or new insights in this book. However, it is an excellent and comprehensive review of a diverse and scattered literature — I know of no other book where this material has been brought together in such an organized and coherent way.

Ronayne interprets the term "science policy" as "policy for science"; there is little discussion of the use of science to inform political decisions about non-scientific issues — what is usually termed "science for policy". He deals with how national governments are or should be organized to support scientific research and development; the theory and practice of determining priorities among areas of research; the pervasive tensions between the claims of the scientific community for autonomy and the demands of society or the political system for accountability; the rationales for government intervention or non-intervention in the industrial innovation system; the role of science policy in fostering industrial innovation and economic growth, and the contribution of basic research to the economy; and the relative merits of centralized and decentralized (or pluralistic) approaches to science policy.

In the accountability versus autonomy

debate, Ronayne concludes that society should determine the broad goals of research and areas of emphasis, while leaving questions of tactics and execution to the scientific community. On the question of scientific priorities, he is sceptical of the value of formalized analytical techniques of assessment, except possibly as an indirect stimulus to more systematic thought about opportunities and needs. He quotes with approval a University of Sussex dictum that "cost-benefit studies should not be undertaken...when the results may be taken seriously". Finally, on centralism versus pluralism, he seems to recommend a middle course, somewhat biased towards a semi-centralized position, in which "countries employ their scientists and technologists in a concerted manner to attack the problems that emerged in the new economic context".

Most of what is available in the literature consists either of advocacy or of multi-authored collections in which no attempt is made to contrast and analyse various perspectives. In addition to providing a comparative view, the book contains excellent descriptions of the government organization for science sponsorship and science policy in most of the major industrialized countries. It should thus provide a valuable textbook for an introductory course in science policy, and it deserves a wide audience. One unique feature is the first chapter, which presents a condensed history of the evolution of science policy from the beginnings of modern science in the seventeenth century; I found this the most original aspect of the book. □

Harvey Brooks is Benjamin Peirce Professor of Technology and Public Policy at Harvard University.

Technology

Nuclear Power Technology. Vol.1: Reactor Technology. W. MARSHALL (ed.). Oxford University Press: 1984. Pp.503. ISBN 0-19-851948-6. £35.

Nuclear Power Technology. Vol.2: Fuel Cycle. By W. MARSHALL. Oxford University Press: 1984. Pp.456. ISBN 0-19-851958-3. £35.

Nuclear Power Technology. Vol.3: Nuclear Radiation. W. MARSHALL (ed.). Oxford University Press: 1984. Pp.363. ISBN 0-19-851959-1. £35.

Numerical and Physical Aspects of Aerodynamic Flows II. TUNCER CEBECI (ed.). Springer-Verlag: 1984. Pp.416. ISBN 3-540-12659-7/0-387-12659-7. DM 152, \$56.70.

The Technology Edge: Opportunities for America in World Competition. By GERARD K. O'NEILL. Simon and Schuster: 1983. Pp.299. ISBN 0-671-44766-1. \$16.95.

Water Sciences and Technology. Vol.15. No.10, Cooling Water Discharges from Coal Fired Power Plants: Water Pollution Problems. No.11, Solid Wastes from Coal Fired Power Plants: Water Pollution Problems. No.12, Airborne Pollutants from Coal Fired Power Plants: Water Pollution Problems. S.H. JENKINS and P. SCHJODTZ HANSEN (eds). Pergamon: 1983. Pbk ISBN 0-08-031024-9, 0-08-031025-7, 0-08-031026-5. Np.

Computer Science

The Commodore 64 Handbook. By PETER LUPTON and FRAZER ROBINSON. Century Publishing: 1984. Pp.248. Pbk ISBN 0-7126-0419-7. £5.95.

Dictionary of Computers, Data Processing and Telecommunications. By JERRY M. ROSENBERG. Wiley: 1984. Pp.614. Hbk ISBN 0-471-87638-0; pbk ISBN 0-471-88582-7. £24.95, \$29.95.

Pascal Applications for the Sciences: A Self-Teaching Guide. By RICHARD E. CRANDALL. Wiley: 1984. Pp.246. Pbk ISBN 0-471-87242-3. Pbk £12.75, \$16.95.

Earth Sciences

Annotated Bibliographies of Mineral Deposits in Europe. Part 1 Northern Europe including examples from the USSR in both Europe and Asia. By JOHN DREW RIDGE. Pergamon Press: 1984. Pp.778. ISBN 0-08-030242-4. £66.50, \$120.

Antarctic Sea Ice, 1973-1976: Satellite Passive-Microwave Observations. By H. JAY ZWALLY et al. National Aeronautics and Space Administration: 1983. Pp.206. ISBN 83-600167. Np.

Assessment of Manganese Nodule Resources: The Data and the Methodologies. Seabed Minerals Series, Vol.1. UNITED NATIONS OCEAN ECONOMICS AND TECHNOLOGY BRANCH. Graham & Trotman: 1982. Pp.79. Np.

Deserts and Arid Lands. FAROUK EL-BAZ (ed.). Martinus Nijhoff: 1984. Pp.222. ISBN 90-247-2850-9. Dfl.115, \$46.

The Dynamic Earth. SCIENTIFIC AMERICAN. W.H. Freeman: 1983. Pp.116. ISBN 0-7167-1611-9. £12.50.

Eddies in Marine Science. ALLAN R. ROBINSON (ed.). Springer-Verlag: 1983. Pp.609. ISBN 3-540-12253-2. DM 120, \$46.60.

English-Russian Dictionary of Applied Geophysics. By B.V. GUSEV et al. Pergamon Press: 1983. Pp.488. ISBN 0-08-028168-0. £27.75, \$50.

Glacial Geology: An Introduction for Engineers and Earth Scientists. N. EYLES (ed.). Pergamon Press: 1983. Hbk ISBN 0-08-030264-5; pbk 0-08-030263-7. Pbk £9.95, \$17.95.

Groundwater Bibliography 3rd edition. By FRITS VAN DER LEEDEN. Water Information Center, The North Shore Atrium, 6800 Jericho Turnpike, Syosset, NY 11791, USA. ISBN 0-912394-19-6. \$18.

Humic Substances: Structural, Photophysical, Photochemical and Free Radical Aspects and Interactions with Environmental Chemicals. By G.G. CHOUDHRY. Gordon and Breach: 1984. Pp.185. ISBN 0-677-06440-3. £44.

Introductory Cartography. By JOHN CAMPBELL. Prentice-Hall: 1984. Pp.406. ISBN 0-13-501304-6. Np.

Mesozoic and Tertiary Geology of Southern Africa. By R.V. DINGLE et al. A.A. Balkema: 1984. Pp.375. ISBN 90-6191-099-4. £24.

Mineralogie: Eine Einführung in die spezielle Mineralogie Petrologie und Lagerstättenkunde. By SIEGRIED MATTHES. Springer-Verlag: 1983. Pp.417. ISBN 3-540-12485-3/0-387-12485-3. DM 58, \$22.50.

Precambrian of South India. Based on the Proceedings of the Indo-US Workshop held at Hyderabad, 12-14 January 1982. S.M. NAQVI and J.J. ROGERS (eds.). Geological Society of India, Bangalore: 1983. Pp.575. Np.

Rock and Mineral Magnetism. By W. O'REILLY. Blackie: 1984. Pp.220. ISBN 0-216-91460-4. £19.50.

Soil Mechanics. 3rd Edn. By R.F. CRAIG. Van Nostrand Reinhold: 1983. Pp.419. Hbk ISBN 0-442-30567-2; pbk ISBN 0-442-30568-0. \$16.95.

Terrigenous Clastic Depositional Systems: Applications to Petroleum, Coal, and Uranium Exploration. By W.E. GALLOWAY and D.K. HOBDA. Springer-Verlag: 1983. Pp.423. ISBN 3-540-90827-7/0-387-90827-7. DM 98, \$38.10.

Volcanic New Zealand. Photographs by ERIC TAYLOR, Text by JAMES COLE. Oxford University Press: 1984. Pp.128. ISBN 0-19-558101-6. £27.50.

Weathering. 2nd Edn. Geomorphology Texts. By CLIFF OLLIER. Longman: 1984. Pp.270. ISBN 0-582-30103-3. £11.50.

Biological Sciences

Abnormal Function Development of the Heart, Lungs and Kidneys: Approaches to Functional Teratology. CASIMER T. GRABOWSKI and ROBERT J. KAVLOCK (eds). Alan R. Liss: 1983. Pp.374. ISBN 0-8451-0140-4. £44.

The Acoustic Sense of Animals. By WILLIAM C. STEBBINS. Harvard University Press: 1983. Pp.168. ISBN 0-674-00326-8. £14.05.

Adaptations, Stress, and Prophylaxis. By FELIX Z. MEERSON. Springer-Verlag: 1984. Pp.329. ISBN 3-540-12363-6/0-387-12363-6. DM 118, \$45.80.

Adaptive Behavior and Learning. By J.E.R. STADDON. Cambridge University Press: 1984. Pp.355. Hbk ISBN 0-521-25699-2; pbk ISBN 0-521-27658-6. Hbk £30, \$59.50; pbk £10.95, \$18.95.

Genetic Engineering: Applications to Agriculture. Beltsville Symposia in Agricultural Research, Vol. 7. L.D. OWENS (ed.). Rowman & Allanheld: 1983. Pp.327. ISBN 0-86598-112-4. \$42.50.

High-Performance Liquid Chromatography of Proteins and Peptides. Proceedings of the 1st International Symposium. M.T.W. HEARN, F.E. REGNIER and C.T. WEHR (eds). Academic: 1983. Pp.267. ISBN 0-12-335780-2. \$27.50.

Human Immunity to Viruses. F.A. ENNIS (ed.). Academic: 1983. Pp.348. ISBN 0-12-239980-3. \$39.

Induced Mutations in Plant Breeding. Monographs on Theoretical and Applied Genetics, Vol.7. By W. GOTTSCHALK and G. WOLFF. Springer-Verlag: 1983. Pp.238. ISBN 3-540-12184-6/0-387-12184-6. DM 98, \$40.50.

Information Sources in Biotechnology. By A. CRAFTS-LIGHTY. Macmillan Press, London/The Nature Press: 1983. Pp.306. Pbk ISBN 0-333-36178-4/0-943818-04-4. £40.

An International Survey of Distributions of Histologic Types of Tumours of the Testis and Ovary. H. STALBERG (ed.). International Union Against Cancer, Geneva: 1983. Pp.353. Pbk ISBN 92-9018-075-7. SwFr. 48, \$24.

Liver Transplantation. R.Y. CALNE (ed.). Grune & Stratton: 1983. Pp.408. ISBN 0-8089-1550-9. £35, \$60.

Microbial Control of Plant Pests and Diseases. Aspects of Microbiology, Vol.7. By J.W. DEACON. Van Nostrand Reinhold: 1983. Pp.88. Pbk ISBN 0-442-30512-5. Np.

Mild Hypertension: Recent Advances. F. GROSS and T. STRASSER (eds). Raven: 1983. Pp.446. ISBN 0-89004-808-8. \$56.

Modern Management of High-Risk Pregnancy. N.H. LAUERSEN (ed.). Plenum: 1983. Pp.508. ISBN 0-306-41306-X. \$59.50.

Muscular Dystrophy: Biomedical Aspects. S. EBASHI and E. OZAWA (eds). Springer-Verlag/Japan Scientific Societies Press: 1983. Pp.302. ISBN 3-540-12342-3/0-387-12242-3/4-7622-6357-5. DM 79, \$34.10.

Oscillations in Mathematical Biology. Lecture Notes in Biomathematics, Vol.51. Proceedings of a Conference held April 1982, Adelphi University. J.P.E. HODGSON (ed.). Springer-Verlag: 1983. Pp.196. Pbk ISBN 3-540-12670-8/0-387-12670-8. DM 32.50, \$12.90.

The Peptides. Vol.5: Special Methods in Peptide Synthesis, Part B. E. GROSS and J. MEIENHOFER (eds). Academic: 1983. Pp.508. ISBN 0-12-304205-4. \$79.50.

Pest Slugs and Snails: Biology and Control. By D. GODAN. Springer-Verlag: 1983. Pp.445. ISBN 3-540-11894-2/0-387-11894-2. DM 196, \$84.50.

Pharmacology, 12th Edn. By G.E. TREASE and W.C. EVANS. Baillière Tindall: 1983. Pp.812. ISBN 0-7020-1007-3. £18.50.

Plant Proteins for Human Food. Proceedings of a European Congress held October 1981, France. C.E. BODWELL and L. PETIT (eds). Martinus Nijhoff: 1983. Pp.471. ISBN 90-247-2856-8. DG 125, \$54.50.

Plasmapheresis: Therapeutic Applications and New Techniques. Y. NOSÉ et al. (eds). Raven: 1983. Pp.462. ISBN 0-89004-980-7. \$72.

Post-Harvest Pathology of Fruits and Vegetables. C. DENNIS (ed.). Academic: 1983. Pp.280. ISBN 0-12-210680-6. £25, \$42.

The Preservation of Fruit and Vegetable Food Products. By S.D. HOLDSWORTH. Macmillan Press, London: 1983. Pp.159. Pbk ISBN 0-333-32292-4. £6.95.

Problems of Antiviral Therapy. C.H. STUART-HARRIS and J. OXFORD (eds). Academic: 1983. Pp.290. ISBN 0-12-674760-1. £17, \$30.

Problems of Drug Dependence, 1982. L.S. HARRIS (ed.). US Department of Health & Human Services: 1983. Pp.552. Np.

Progestogens in Therapy. Sero Symposia Publications from Raven Press, Vol.3. G. BENAGIANO, P. ZULLI and E. DICZFALUSY (eds). Raven: 1983. Pp.280. ISBN 0-89004-856-8. \$68.50.

Progress in Clinical Immunology: The Role of Chemical Mediators and Cellular Interactions. Monographs in Allergy, Vol.18. M. RICCI and G. MARONE (eds). Karger: 1983. Pp.314. ISBN 3-8055-3697-6. SwFr. 164, DM 196, \$98.25.

Progress in Retinal Research. Vol.2. N.N. OSBORNE and G.J. CHADER (eds). Pergamon: 1983. Pp.330. ISBN 0-08-030773-6. £40, \$72.

Protection Against Trichothecene Mycotoxins. Committee on Protection Against Mycotoxins. National Academy of Sciences Press: 1983. Pp.227. Pbk ISBN 0-309-03430-2. \$17.95.

Protective Agents in Cancer. D.C.H. MCBRIEN and T.F. SLATER (eds). Academic: 1983. Pp.320. ISBN 0-12-481770-X. £18, \$30.

Recombinant DNA Applications to Human Disease. Banbury Report 14. C.T. CASKEY and R.L. WHITE (eds). Cold Spring Harbor Laboratory: 1983. Pp.375. ISBN 0-87969-214-6. \$55 (US), \$66 (elsewhere).

Recombinant DNA: A Short Course. By J.D. WATSON, J. TOOZE and D.T. KURTZ. W.H. Freeman: 1983. Pp.260. Hbk ISBN 0-7167-1483-3; pbk ISBN 0-7167-1484-1. Hbk £24.95; pbk £14.95.

Rhythms in Biology and Other Fields of Applications: Deterministic and Stochastic Approaches. Lecture Notes in Biomathematics, Vol.49. Proceedings of a Meeting held September 1981, France. M. COSNARD, J. DEMONGEOT and A. Le BRETON (eds). Springer-Verlag: 1983. Pp.400. Pbk ISBN 3-540-12302-4/0-387-12302-4. DM 56, \$22.30.

Selected Topics in Preventive Cardiology. Ettore Majorana International Science Series, Vol.12. A. RAINERI and J.J. KELLERMANN (eds). Plenum: 1983. Pp.275. ISBN 0-306-41375-2. \$45.

Sexually Transmitted Diseases in Homosexual Men: Diagnosis, Treatment and Research. D.G. OSTROW, T.A. SANDHOLZER and Y.M. FELMAN (eds). Plenum: 1983. Pp.272. ISBN 0-306-41337-X. \$35.